HEXAHYDROPYRIMIDINES AS MASKED SPERMIDINE VECTORS IN DRUG DELIVERY AND AS REAGENTS IN THE SYNTHESIS OF H-SHAPED OCTACOORDINATE ACTINIDE LIGANDS FOR HUMAN AND ENVIRONMENTAL DECONTAMINATION

By

HOWARD WAYNE SELIGSOHN

A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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1987

Through their love,

patience,

understanding,

inspiration,

and constant support,

my mother and father have contributed

immeasureably to my career. In recognition of this, I proudly dedicate this

dissertation to them.

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I would like to extend my sincere gratitude to my major advisor, Dr. Raymond J. Bergeron, for all his support, patience and understanding throughout my studies. I also thank my committee, Drs. Richard Strieff, Kenneth Sloan, Merle Battiste, and Margaret James for their contribution to this work. To Dr. James Navratil and the Rocky Flats research group I extend my thanks for the plutonium data. Deserving special thanks are Drs. Nelson Scarborough and Steven Prudencio and Ms. Kady Crist for their surgical expertise, Diana Tukalo for her synthetic efforts, and Micheal Ingeno for all of his help with my in vitro experiments. Lastly, thanks to all of my good friends and family who have helped me through the difficult times. I could not have made it without your support.

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Chairman: Dr. Raymond J. Bergeron
Major Department: Medicinal Chemistry

The cellular uptake of several polyamines is evaluated in terms of their intermolecular hydrogen bonding and their charge at physiological pH. N-(4-Aminobutyl)hexahydropyrimidine and N-(3-aminopropyl)hexahydropyrimidine are shown to compete with spermidine for uptake by L1210 cells. This observation is in keeping with the idea that spermidine may adopt a hydrogen-bonded cyclic structure in the course of transport. Furthermore, the differences in the ability of spermidine, homospermidine, and norspermidine to utilize the spermidine uptake apparatus of L1210 cells is related to the protonation state of the amines. These states are calculated for each triamine from measured pK_a data. The hexahydropyrimidines used in the polyamine uptake sudies were next used in the synthesis of octacoordinate catecholamide H-shaped ligands. These chelators were tested for their ability to remove actinides from aqueous solution, human plasma, and rats. H-Shaped ligands removed more than 99.9 percent of the thorium from a 0.32 μM aqueous solution. In addition, approximately 30 percent of the

CHAPTER I

INTRODUCTION

In recent years, scientists have become increasingly aware that polyamines play an important role in cellular metabolic processes. Considerably increased levels of ornithine decarboxylase, the rate-limiting enzyme in polyamine biosynthesis, is an apparent "priming" event for growth in a variety of systems (1). Many neoplasias have been shown to concentrate polyamines intracellularly (2, 3, 4). Polyamine biosynthesis and intracellular accumulation are intimately associated with rapid cell proliferation. The increase in intracellular spermidine has been shown to correlate well with the increase in intracellular RNA (5). Elevated polyamine levels in urine have been detected in tumor-bearing patients (6). Bergeron and Porter have demonstrated that polyamine-deficient L1210 cells divide much more slowly than control cells (7, 8, 9, 10). Thus it appears that an intracellular polyamine demand, polyamine biosynthesis and intracellular accumulation are intimately associated with rapid cell proliferation.

Since neoplastic tissues exhibit a high polyamine requirement when compared to normally differentiated tissues, the idea of using polyamines as vectors for delivering antineoplastics is an attractive one. If one were to design a polyamine derivative which possessed antineoplastic properties, and could also utilize the polyamine transport apparatus for cellular uptake, drug selectivity may be improved. Such a derivative could, of course, be taken up into normal tissue as well as rapidly dividing tissue. However, since neoplastic tissues exhibit high intracellular polyamine demand and accummulation relative to normal tissues, selective toxicity should be observed.

The design of polyamine antiproliferatives must be based upon a careful consideration of the polyamine transport apparatus. Implicit in this is an understanding of the solution conformations attained by substrates of the polyamine transport apparatus. With these

considerations in mind, several experiments were designed to ascertain the structural boundaries for substrates of the polyamine transport apparatus. More exactly, we wanted to know the degree to which a polyamine could be derivatized without significantly affecting uptake by the polyamine transport apparatus. Previous studies led us to postulate the importance of an intramolecularly hydrogen bonded structure (11, 12). To test this hypothesis, hexahydropyrimidine derivatives were prepared (13, 14) and found to be effective inhibitors of spermidine uptake.

These hexahydropyrimidines, as well as having interesting biological activity, turned out to be very useful as synthetic reagents, leading to a variety of actinide chelating agents which are based on the structures of naturally-occurring siderophores possessing a polyamine backbone.

Actinide contamination in humans does not represent a major health hazard because of the federal and self-imposed safety precautions adopted by those in the nuclear industry. Still, the potential for contamination exists and incidents involving human exposure and contamination have occurred (15, 16, 17). Environmental contamination is more common than internal human contamination. In a laboratory situation, when contamination occurs, trained personnel are on hand to contain the hazard and treat the problem as is necessary. When contamination occurs outdoors, the problem is not easily contained and is, therefore, more serious. Not only can water supplies, soil, vegetation, and livestock become contaminated, but curious individuals--simply by their presence--may unknowingly contaminate themselves at the site of an accident as well. One can imagine how easily a localized contamination could spread, since the danger cannot be seen.

Environmental and external human decontamination ultimately generate a large volume of aqueous solution which contains very low levels of hazardous actinide. This presents a problem in terms of disposal. At the present time most of this water is buried in uninhabited regions of the country, but this is only a temporary solution. Eventually, the integrity of the storage containers may fail, leading to new contamination.

If, on the other hand, this water could be exposed to an actinide-specific ligand and the complex removed by some physical means, the water could be decontaminated. More specifically, if a ligand were bound to a polymer backbone, a resin with a high affinity for actinides would be the result. This could be used to concentrate the contamination onto a small amount of solid resin. In this way, the complex would be converted into a form from which the metal could be recovered--by pH manipulation, ashing, etc. A resin-bound ligand would be practical for many other aspects of decontamination as well. For instance, this type of ligand could be very useful as a tool for dialysis. An insoluble ligand could also be used for prevention of absorption of ingested actinides.

Environmental contamination can lead to both internal and external human contamination (due to our irresistible urge to touch things) and internal human contamination. Since actinides can invade the body via several routes, such as the lungs (18), GI tract (19) or open wounds (20), the degree to which a particular organ or body compartment is contaminated will depend on the site of entry (21, 22). For example, lung contamination is observed in cases of actinde inhalation. Once absorbed, actinide deposition initially occurs in many tissues of the body: liver, kidneys, muscle tissue, lungs, heart, testes, and bone (23). With time, the metal continually migrates to the liver and bone, while other soft tissue levels decrease. Once in these sites the metal is much more difficult to remove. Clearly, the sooner a contaminated individual can receive treatment, the easier the decontamination process.

No adequate therapeutic treatment exists for persons internally contaminated with actinides. Contamination of humans is generally external and most often the metal is removed long before absorption can occur, minimizing the seriousness of the problem. However internal contamination does occur and is more serious, owing to the limited therapeutic options.

The treatment of choice is currently diethylene triamine pentaacetic acid (DTPA) chelation therapy, figure 1, but DTPA has several problems associated with it. In addition to the fact that the drug removes an insufficient quantity of actinide (24, 25, 26), DTPA has toxicity problems,

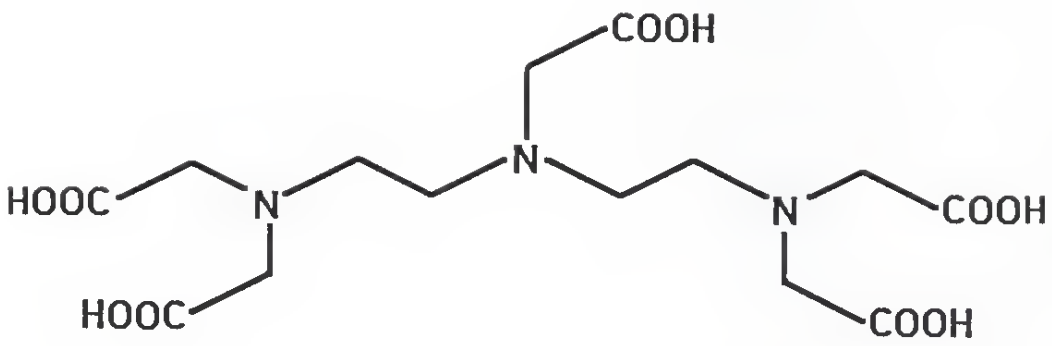
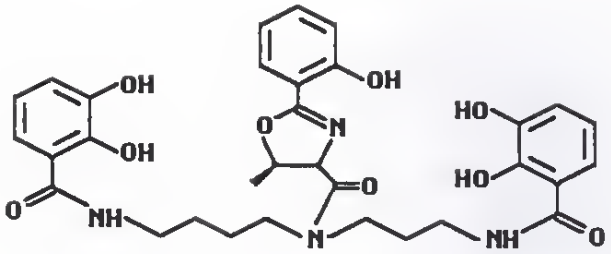


FIGURE 1
DTPA (Diethylene Triamine Pentaacetic Acid).

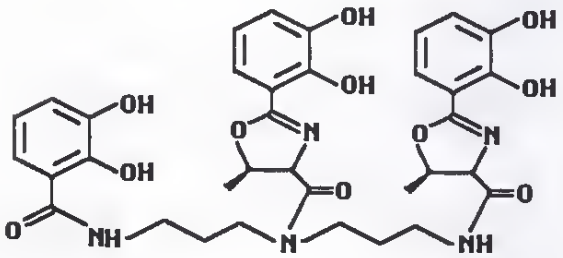
most of which are associated with its ability to sequester and remove zinc from the body (27, 28). Although the latter difficulty has been overcome by administering the drug as its zinc complex (29), the former problem remains--DTPA alone simply cannot remove an adequate amount of actinide. For example, when DTPA was administered to rats intravenously over a three hour period 24 hours after intramuscular injection of plutonium nitrate or citrate, a dose of 120 μmoles/kg was necessary to achieve excretion of only 20 percent of the injected dose, relative to control animals (25). It is unlikely that this problem can be solved even by modification of administrative routes; e.g., continuous infusion (30). Because of its highly polar zwitterionic nature, DTPA is poorly absorbed from the bloodstream and is rapidly excreted in the urine (N. L. Scarborough, private communication), implying that DTPA's inadequacy is a result of its volume of distribution (31, 32). To obviate the shortcomings of DTPA, more lipophilic catecholamide chelators were prepared (33, 34, 35).

Some of the most stable metal complexes known are formed between catecholamide ligands--siderophores--and iron(III). For example, the parabactin-iron(III) formation constant is 10^{48} M^{-1} (K. N. Raymond, private communication), parabactin being a hexacoordinate catecholamide ligand with a spermidine backbone. Plutonium(IV) and iron(III) are similar with respect to the chemical properties responsible for complex stability; i.e., high charge, small ionic size, and high acidity. For example, Pu(IV) has a charge-to-radius ratio of 444 e/μm as compared to 460 e/μm for Fe(III) (36). Consequently the coordination chemistry of the actinide metals and iron is very similar. This similarity extends to their biological properties as well. For example, both metals are bound tightly to transferrin (37, 38, 39) and ferritin (40) and associate with the trabecular bone. Due to plutonium's larger ionic radius, it forms more stable complexes with octadentate ligands, in contrast to Fe(III), which forms very stable chelates with hexacoordinate ligands, figure 2.

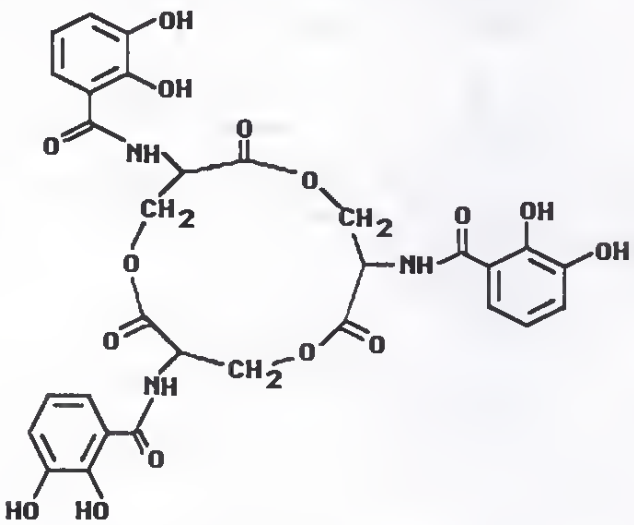
Based on the above similarity, researchers have used their experience with iron-specific hexacoordinate chelators to design of octacoordinate actinide-specific chelators: the design of



Parabactin



Vibriobactin



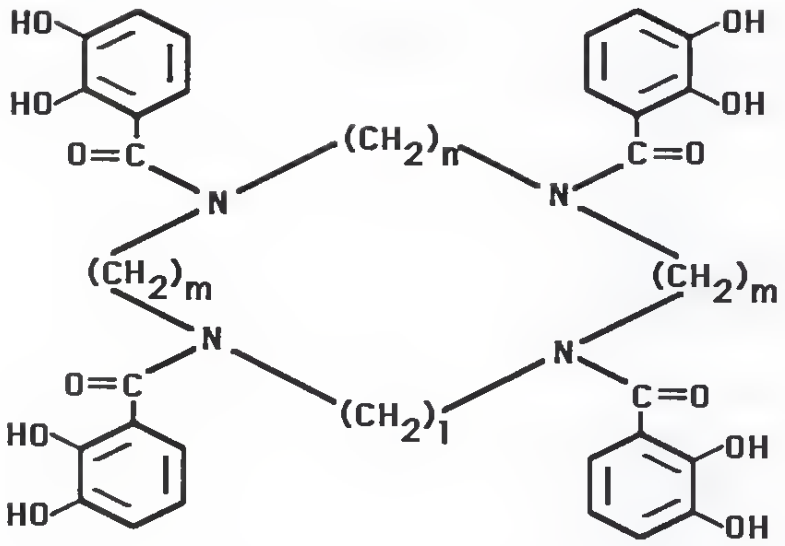
Enterobactin

FIGURE 2
Naturally-Occurring Siderophores.

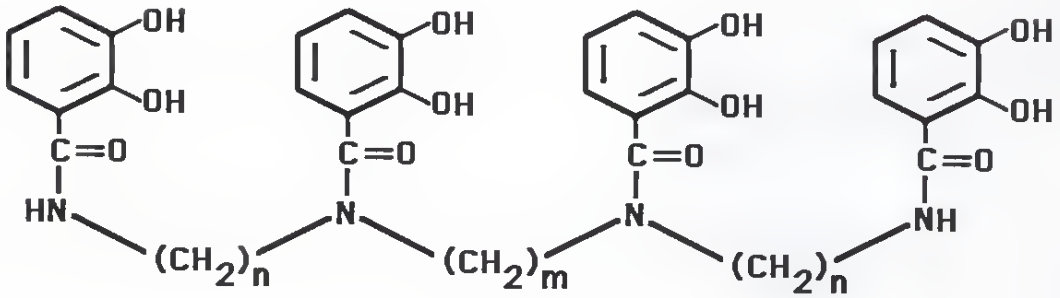
actinide chelators is based on the assumption that octacoordinate ligands represent the optimum situation for binding actinides, and that actinides will form the best chelate with the catechol molety. From this reasoning emerged a variety of effective cyclic and linear octacoordinate catecholamide ligands, referred to as CYCAM (cyclic catecholamide)and LICAM (linear catecholamide), figure 3 (33, 34, 41). These compounds have been shown to stimulate actinide excretion (21, 25, 30, 32, 33, 34, 42, 43, 44). A derivative of LICAM in which each catechol has a 4-carboxyl group attatched, LICAMC, was the most promising of these, figure 4. It was found that LICAMC did access different body compartments than DTPA (42). For example, DTPA removed much less of the actinide associated with the skeleton than LICAMC. Unfortunately, LICAMC shows signs of nephrotoxicity, as indicated by a 300 percent increase in the amount of plutonium deposited in this organ, relative to controls.

Since no one therapeutic agent, such as DTPA or LICAMC, for example, is likely to be able to access all sites of actinide deposition, it is unreasonable to expect that a single drug can serve to remove all of a toxic metal from a contaminated individual. In addition, even if a chelator can remove all of a particular actinide, it may be ineffective at removing other actinides. For example, Mays et al. found that DTPA removed 70 and 60 percent of the deposited plutonium and americium, respectively, from beagles (30). At the same dosage, LICAMS and LICAMC were able to remove 86 and 88 percent of the deposited plutonium, respectively, but these same chelators were only able to remove 33 and 28 percent of the injected americium. Unfortunately, LICAMS also shows signs of toxicity in the form of renal hemmorhage, edema, and an abrupt rise in blood urea nitrogen (45). These observations suggest that the answer to chelation therapy lies in the application of several non-toxic chelators, each with a different volume of distribution, so that all actinides are accessable to at least one agent.

Volf came to the same conclusion, leading him to measure the ability of the combination of DTPA and LICAMC to stimulate plutonium excretion relative to stimulation by each chelator individually (43). He found that the combination did show increased removal, but LICAMC



l,m,n-CYCAM



n,m,n-LICAM

FIGURE 3
Synthetic Catecholamide Ligands.

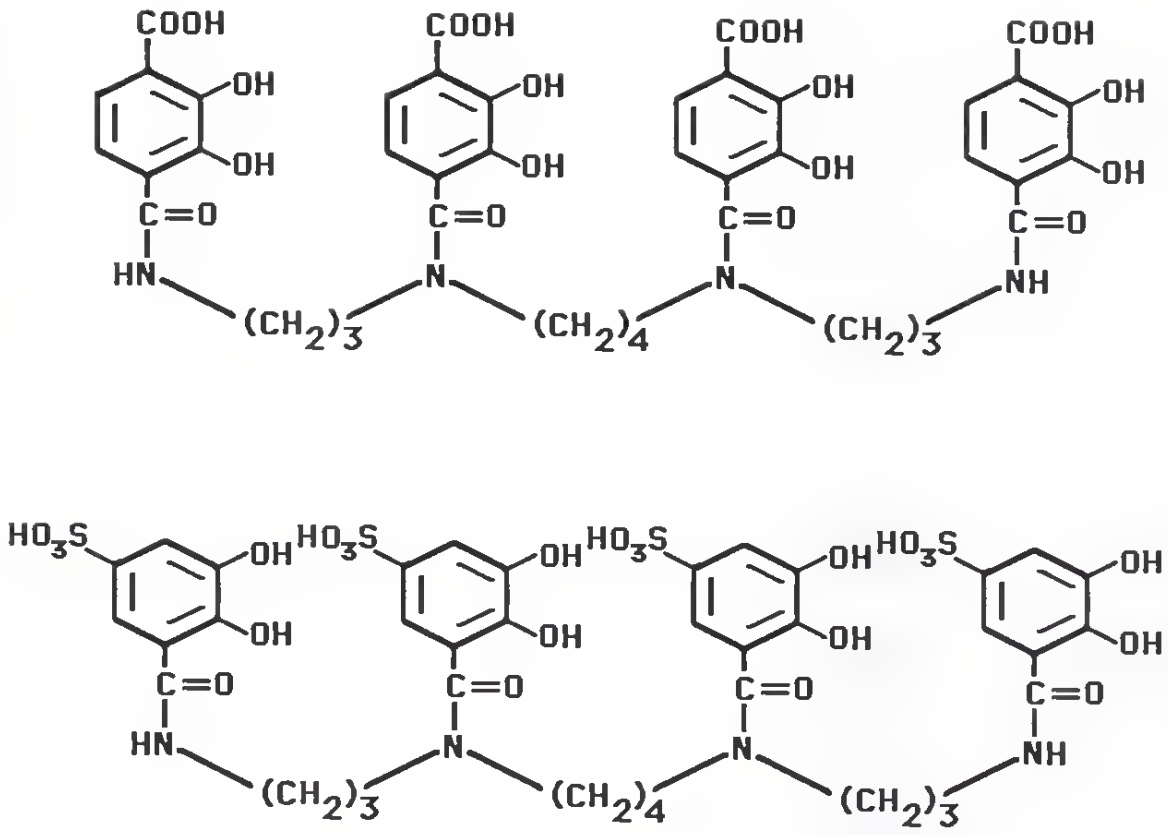


FIGURE 4
LICAMC (Linear Catecholamide, Carboxylated) and
LICAMS (Linear Catecholamide, Sulfonated).

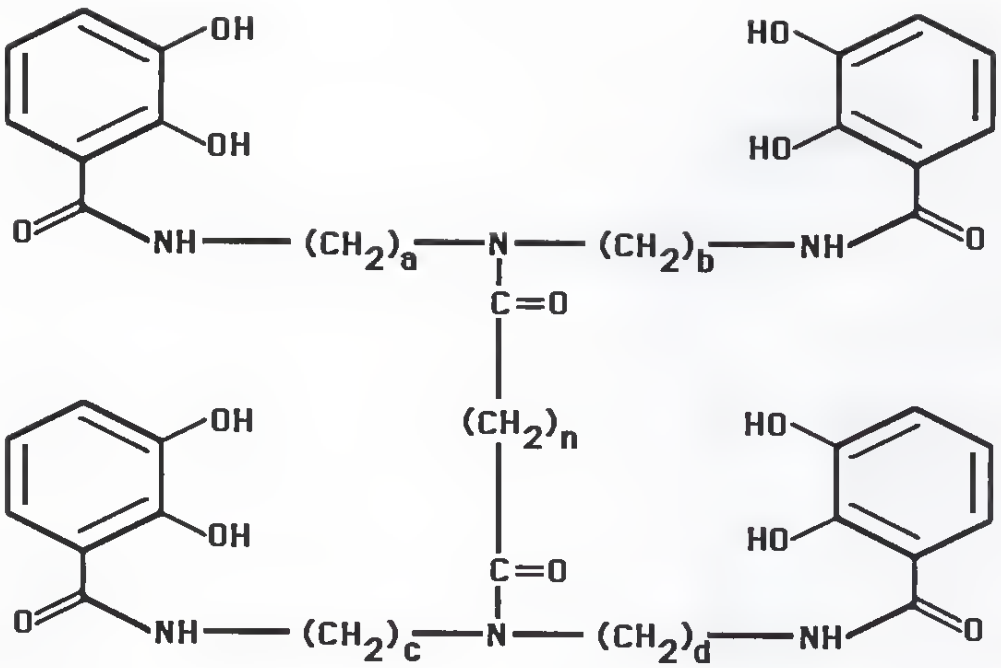
toxicity was still evident. Mays examined plutonium excretion by DTPA and/or LICAMS. His results indicate that the combination of chelators is as effective as DTPA alone at americium removal, and as effective as LICAMS alone at plutonium removal (30). Therefore combination chelator therapy represents a practical approach to the problem of total actinide decorporation in man.

In hopes of alleviating the problems stated above, a series of new octacoordinate catecholamide chelators for application to both biological and environmental actinide decontamination was designed, figure 5. We have developed a structurally novel system referred to as H-shaped ligands, in reference to the unusual structure of their backbone. Each point of the H is bound to a catechol moiety. Our goal was to synthesize a series of non-toxic ligands, each with its own unique backbone, which possessed a selective affinity for actinide metals, and could acess more or different body compartments than DTPA.

This series of ligands is not carboxylated; the carboxyl groups are one potential source of LICAMC's observed toxicity. Additionally, H-shaped ligands are based upon a spermidine backbone rather than spermine. Based upon experience with N^1,N^8-bis(2,3-dihydroxy-benzoyl)spermidine (Cpd II)--a probable product of metabolism, if metabolism occurs--the H-shaped ligand backbone should also be non-toxic (46).

High yield syntheses were designed and carried out to give the thirteen symmetric and assymmetric model catecholamide chelator systems in figure 5. The aim of the design was to synthesize a series of ligands whose geometries and lipophilic properties differed in order that the binding geometry of the ligand to a specific metal could be optimized, and in order that the ligands would exhibit differences in lipophilicity which would carry them into different body compartments.

By way of analogy, imagine that each metal and each ligand of interest are a sphere and hand, respectively, and that the optimum geometry of the chelate, or "grip", is achieved when



Symmetric Ligands

cpd	n	a	b	c	d
1a	2	3	3	3	3
1b		4	4	4	4
1c		3	4	3	4
1d	3	3	3	3	3
1e		4	4	4	4
1f		3	4	3	4
1m	4	3	4	3	4

"Mixed" or
Asymmetric Ligands

cpd	n	a	b	c	d
1g	2	3	3	3	4
1h		3	3	4	4
1i		3	4	4	4
1j	3	3	3	3	4
1k		3	3	4	4
1l		3	4	4	4

FIGURE 5
H-Shaped Ligands.

only the catechol moieties, or "fingertips," of a hand can comfortably hold a "sphere." If a hand is small in relation to the sphere of interest, the grip will be weak. Conversly, as the hand grows large in relation to the sphere, it becomes difficult to hold the sphere with the tip of every finger. Clearly, a ligand which binds, or grips, metals indiscriminately would quickly be rendered inactive, since other ions, or spheres, present would soon saturate the metal binding sites. It was demonstrated that H-shaped ligands do indeed exhibit a selective affinity for actinides in aqueous solution, human plasma, and rats.

CHAPTER II

H-SHAPED OCTACOORDINATE ACTINIDE LIGANDS FOR HUMAN AND ENVIRONMENTAL DECONTAMINATION

Background

High yield syntheses were designed and carried out on the thirteen symmetric and asymmetric model catecholamide chelator systems in figure 5. The aim ot the synthetic program was to design and synthesize a series of ligands whose geometries and lipophilic properties differed. It successful, the binding geometry ot the ligand to a specific metal could be optimized, and the ligands would exhibit ditterences in lipophilicity, carrying them into different body compartments. These two issues are relevent in the tollowing ways.

It is clear that different actinide metals will have ditterent optimum ligand binding geometries. For example a ligand which binds plutonium may not bind americium as effectively. However, whether the metals' chelation geometries are significantly different remains to be and must be established.

With regards to lipophilicity, because the distribution volumes of the actinide metals are not equivalent throughout the various tissues (47), designing ligands having different distribution volumes is essential if all actinide pools are to be acessed. This is critical in the development ot a chemotherapeutic system for total actinide removal. In the case ot LICAMS and DTPA, when only LICAMS was used, only americium was effectively excreted. Conversely, if only DTPA was used, only plutonium was excreted effectively. When administered in combination, however, both actinide metals were effectlvely excreted. This illustrates how a combination of chelators can effectively eliminate all of the actinide burden from an animal where single chelators could

not. Of course one hopes that a single drug will remove all of the toxic metals; however, this is unlikely.

During the synthesis of these compounds, it became evident that H-shaped ligands are only slightly soluble in neutral or acidic aqueous solutions. If, however, one could lower the pK_a's of the catechol moieties then the pH range over which the ligand is soluble would expand. Raymond and his associates have made several attempts to do this involving carboxylation and sulfonation of the aromatic portions of his catecholamide ligands (33, 34). These derivatives did not exhibit a very impressive change in water solubility. In fact, the final products were isolated by recrystallization from water. With this same goal in mind, N,N-diethyl-(2,3-dihydroxy-5-nitro) benzamide was prepared for puposes of potentiometric titration, along with its parent compound N,N-diethyl-(2,3-dihydroxy)benzamide. If the pK_a's of this nitrated monomeric derivative are substanially lowered, relative to those of the unsubstituted monomer, then the water solubility of the tetranitro H-shaped ligand should be greatly improved relative to the unsubstituted ligand. In addition to increasing water solubility, the visible absorption spectrum of the nitro monomer was found to be pH-dependent, implying that a nitro ligand could be a useful analytical tool for metal binding. It was not our intention to apply this ligand to biological decontamination since nitro compounds, in general, are notoriously toxic. This was observed with CYCAM-NO_2 (44). However, it may be useful for environmental decontamination, increasing the pH range over which the ligand is effective.

During the course of these studies, it became evident that an insoluble form of chelator would be very useful, possible applications being water decontamination, prevention of gastrointestinal actinide absorption, dialysis, and prevention of percutaneous absorption. Thus, a resin-bound catecholamide was prepared. If contaminated water was passed through a column containing the resin-linked ligand, the contamination would be concentrated onto the column material, effectively decontaminating the water. If inert to the body's digestive processes, a resin-bound ligand could sequester any ingested actinides and prevent their

absorption into the body. Although gastrointestinal absorption is not a problem in the case of PuO_2, other forms of plutonium are absorbed and retained (48). The bound metal-ligand complex would then be excreted intact via the feces. This same type of system could be used to dialyze actinides without exposing the patient to systemic chelators. It should be pointed out that we are not trying to synthesize a single derivatized resin to perform all of these feats. We intend only to create a single such derivative in order to determine if matrix-linked ligands are a useful concept. If the concept works, different resin materials can be investigated to optimize the effectiveness of each application.

Synthesis

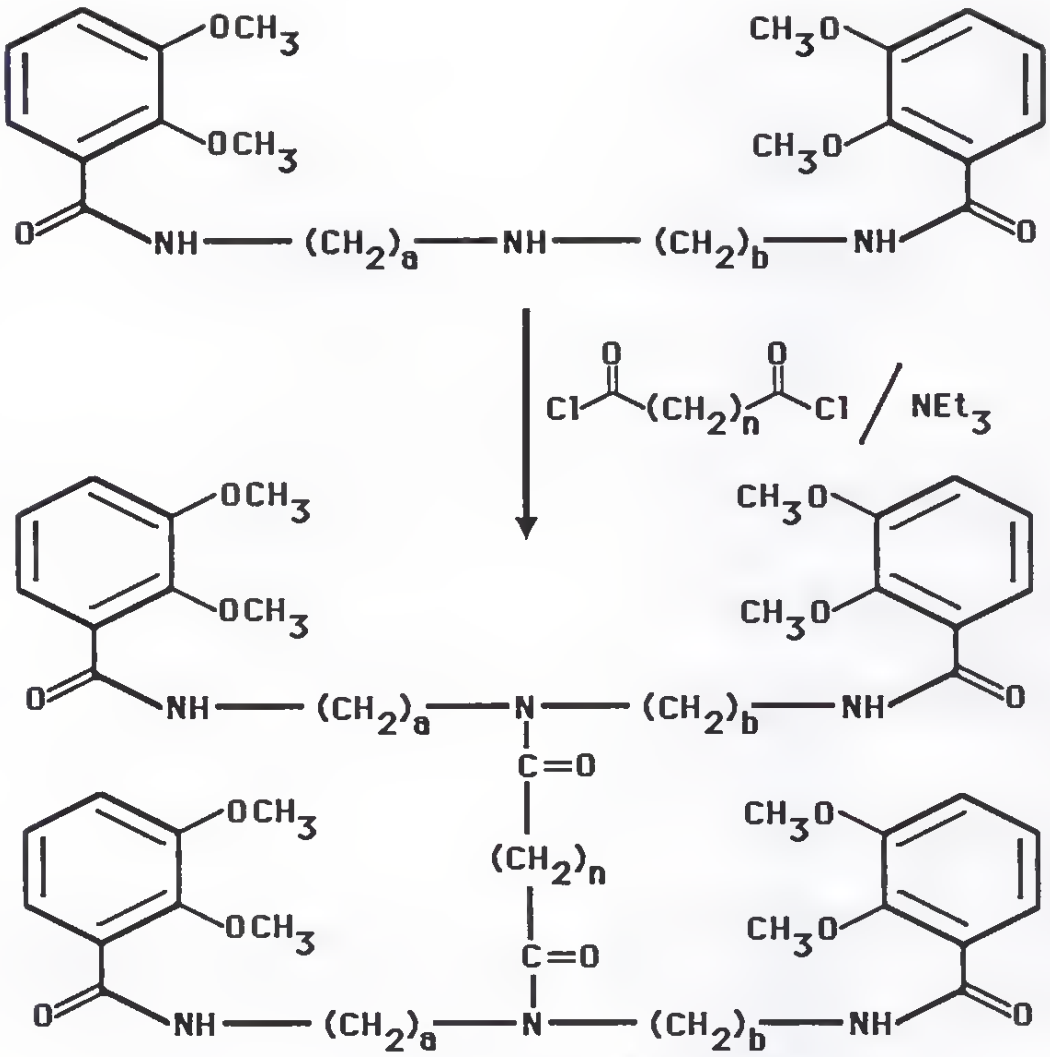
Catecholamide Ligands

Parent catecholamides

It should be pointed out that all syntheses were designed to facilitate modification of the molecule. This is critical in drug design, as structural changes are frequently required in order to improve on pharmacological behavior. Without such flexibility, one would be faced with starting from the beginning--i.e., developing a new synthetic sequence for each structural analog--rather than simply introducing a modified reagent into the present design.

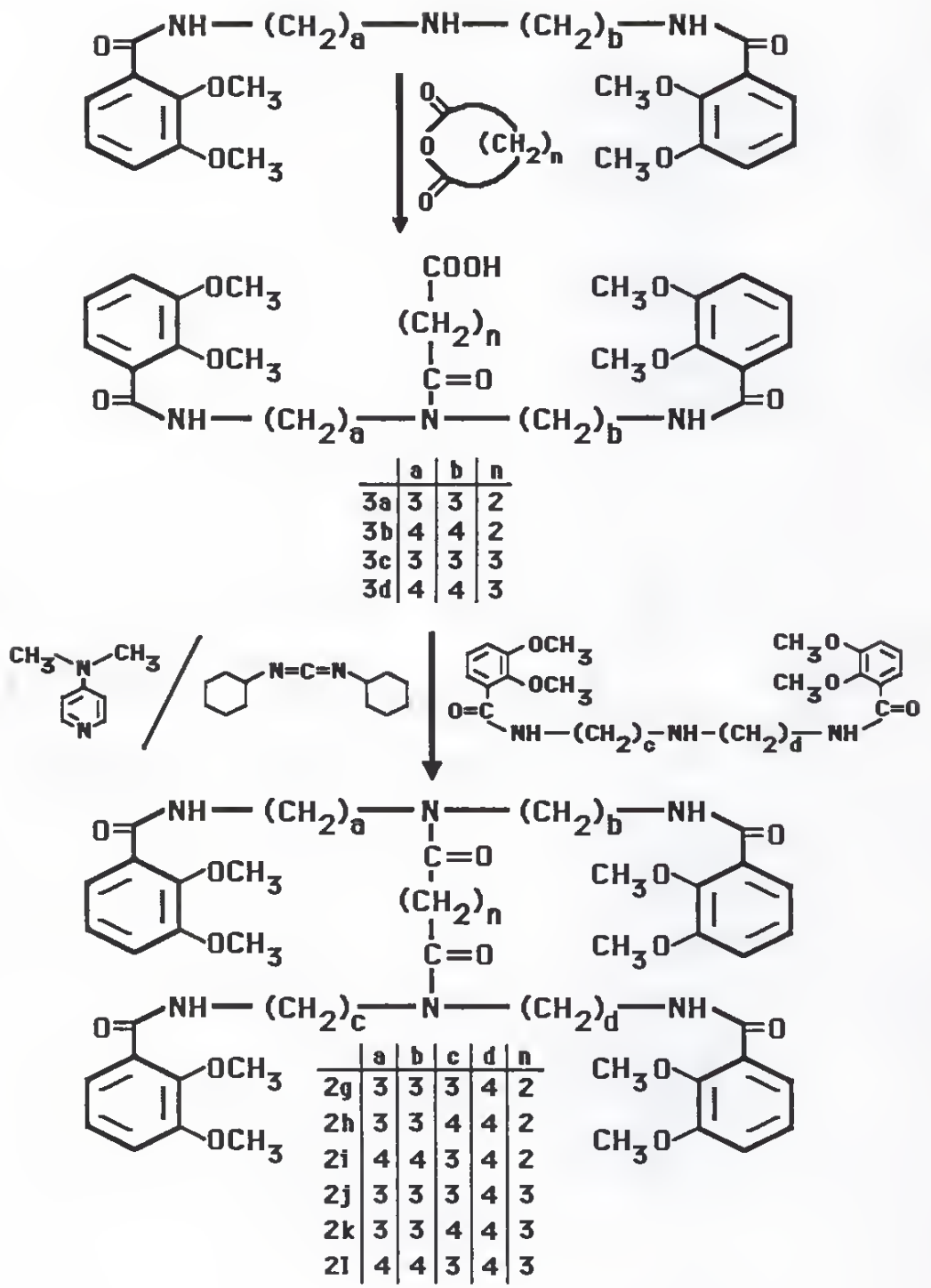
Note that the thirteen analogs shown in figure 5 fall into two classes--symmetric (1a-f and 1m) and asymmetric or "mixed" ligands (1g-l). Symmetric ligand precursors (2a-f and 2m) were synthesized by coupling an appropriate acid dichloride (succinyl, glutaryl, or adipoyl) with two equivalents of either N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine, norspermidine, or homospermidine, figure 6.

In the case of an asymmetric ligand, one must selectively acylate each end of a diacid with a different spermidine derivative, necessitating a two-step process; i.e., the stepwise addition of each amine derivative. This is achieved by the aminolysis of succinic or glutaric anhydride (49) by the nor- or homospermidine derivative, figure 7. The "half-acids" thus produced (3a-d) can next be coupled to a different spermidine derivative, yielding methylated asymmetric ligand



cpd	a	b	n
2a	3	3	2
2b	4	4	2
2c	3	4	2
2d	3	3	3
2e	4	4	3
2f	3	4	3
2m	3	4	4

FIGURE 6
Synthesis for Symmetric Ligands.



	a	b	n
3a	3	3	2
3b	4	4	2
3c	3	3	3
3d	4	4	3

	a	b	c	d	n
2g	3	3	3	4	2
2h	3	3	4	4	2
2i	4	4	3	4	2
2j	3	3	3	4	3
2k	3	3	4	4	3
2l	4	4	3	4	3

FIGURE 7
Synthesis for Assymmetric Ligands.

precursors (2g-l). The removal of the methyl protecting groups results from the action of boron tribromide, figure 8.

Nitro derivatives

Initially, synthesis of a tetra-nitro derivative of an H-shaped ligand was attempted via the tetra-BOC precursor 4d shown in figure 9. This method yielded the desired compound, but required many steps; thus the total synthesis was very time consuming. Also, one must acylate each amine with the same group; we ultimately hoped to be able to place a different acyl group on each amine so that we could "fine tune" the biological properties of the drug. To make this synthesis more cost and time efficient and more flexible, other synthetic methods were sought.

We decided to utilize the synthesis used for symmetric ligands, figure 10. This synthesis was expected to proceed without difficulty, based upon the experience of other similar molecules. The problems, we expected, would occur in the synthesis of the tetramethoxy precursor 6. Until this time, literature methods (50, 51) were used to prepare tetramethoxy precursors, figures 11 and 12. This methodology is unsuitable for the synthesis in question. Although 2,3-dimethoxy-5-nitrobenzoyl chloride is easily prepared and smoothly acylates N^4-benzyl norspermidine, the hydrogenolysis conditions necessary for cleavage of the N-benzyl protecting group would surely reduce the aromatic nitro groups to nitrosos and/or amines. Therefore another protecting group was sought. After several other groups were investigated, the tetramethoxy precursor 6 was synthesized using the formaldehyde adduct of norspermidine, N-(3-amino-1-propyl)hexahydropyrimidine, figure 13. To this hexahydro-pyrimidine--prepared by the method of McManis (13, 14)--was added two equivalents of 2,3-dimethoxy-5-nitrobenzoyl chloride, giving 8. The deprotection was performed by several different aldehyde trapping agents, but dimedone (5,5-dimethyl dihydroresorcinol) proved to be the most reproducible and versatile method, effectively deprotecting a wide variety of hexahydropyrimidine derivatives in high yield. Compound 6 was reacted with succinyl dichloride to yield the tetranitro precursor 5a, figure 10. It seems safe to assume that the asymmetric

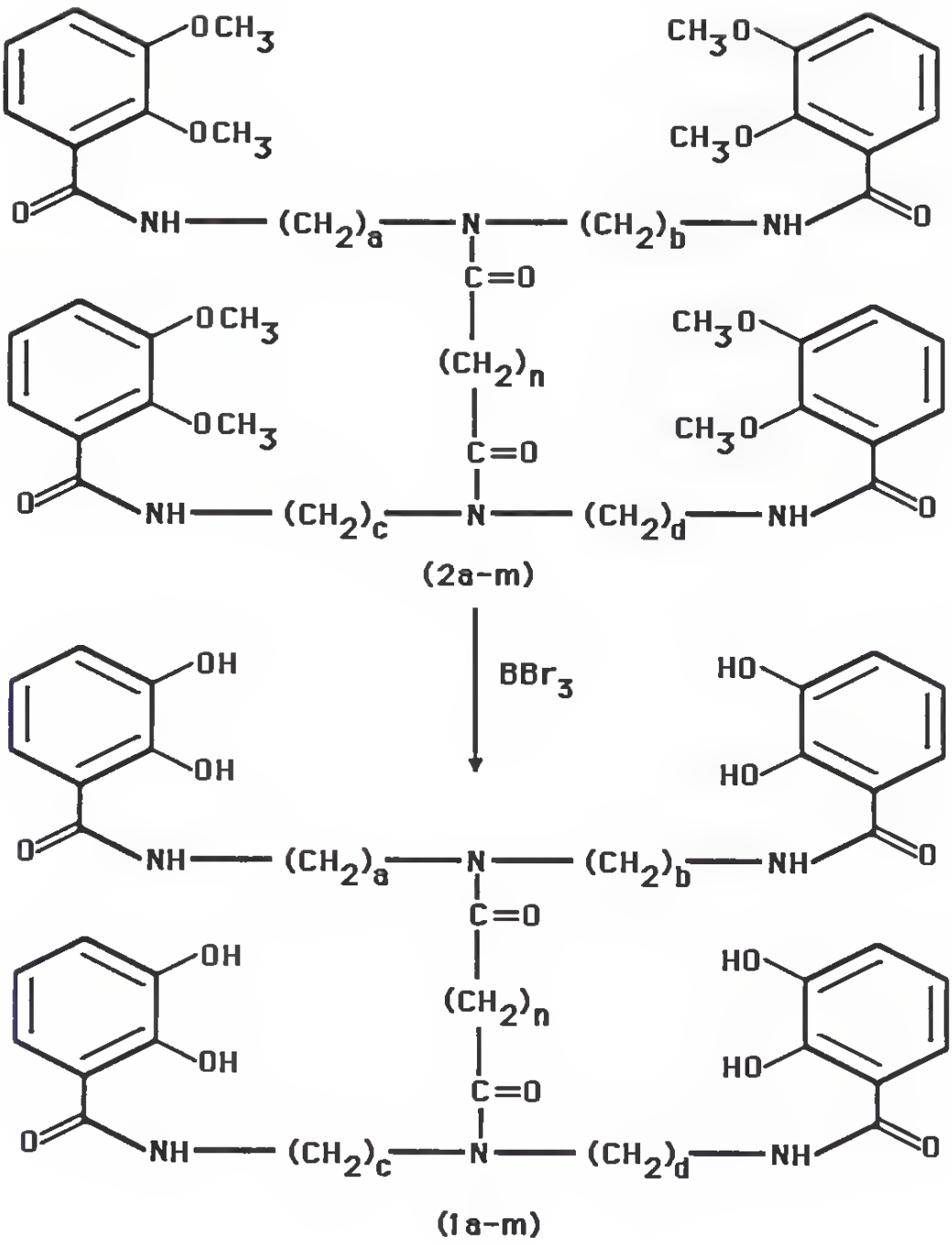


FIGURE 8
Removal of Protecting Groups.

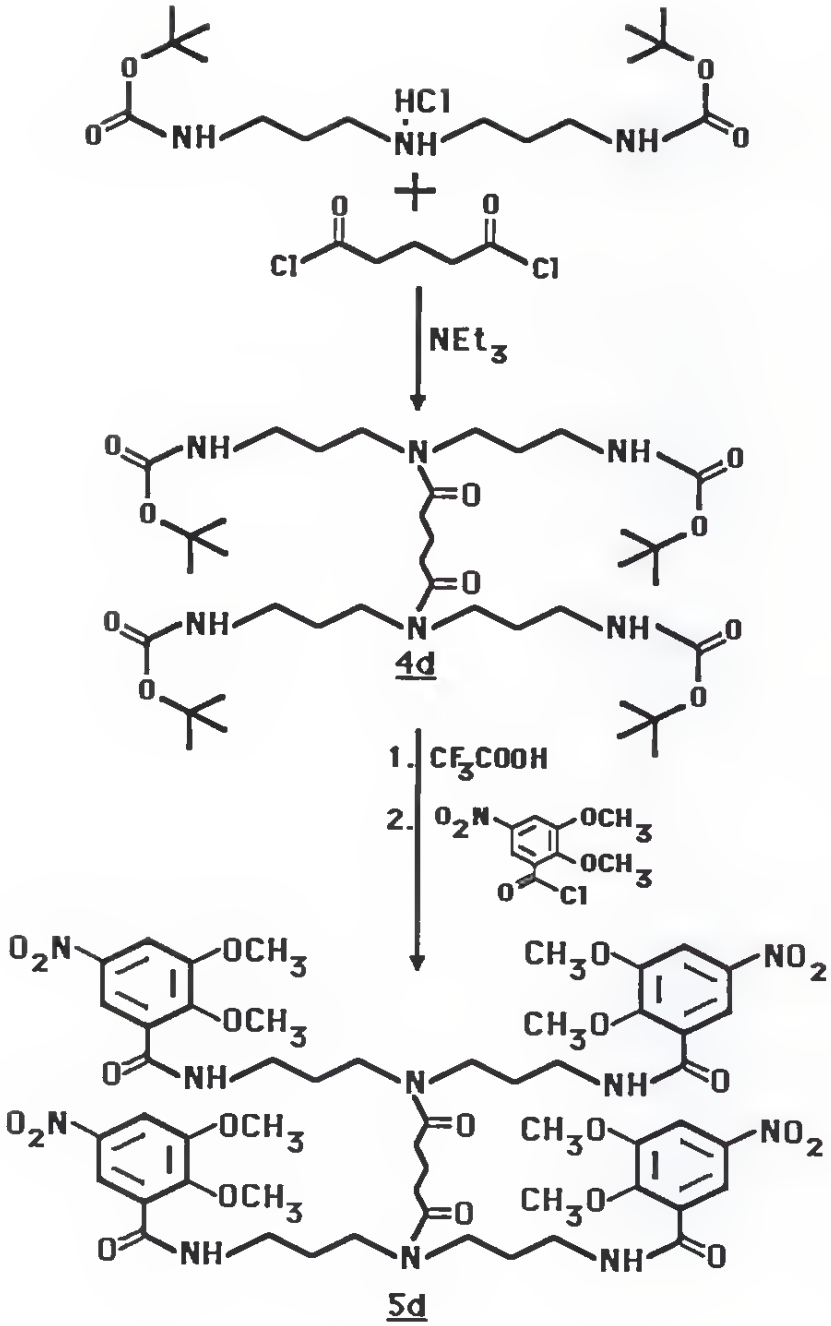


FIGURE 9
Synthesis for Tetra Nitro Ligand.

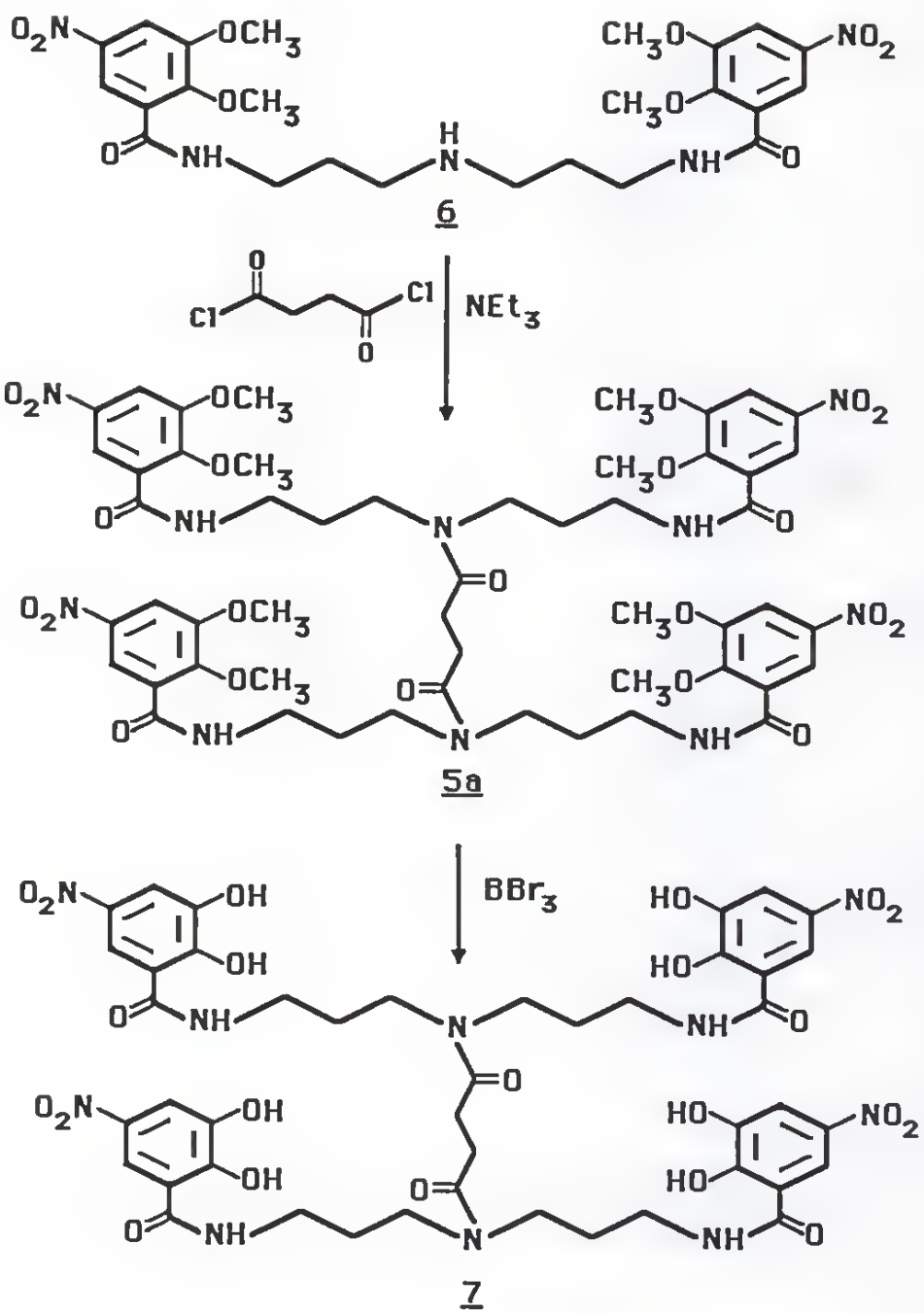


Figure 10
Alternate Synthesis for Tetra Nitro Ligand.

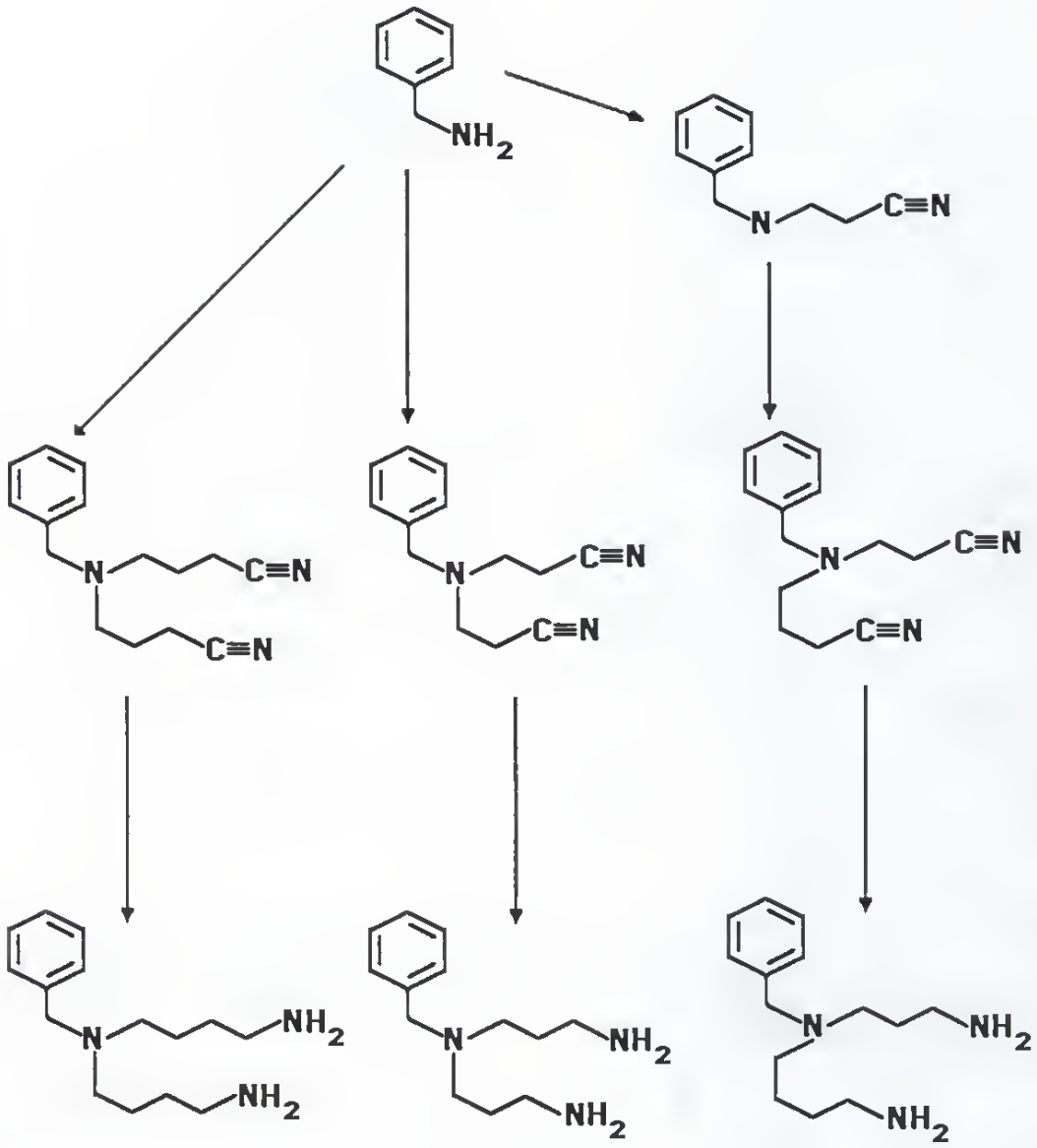


FIGURE 11
Synthesis for N^4-Benzyl Triamines.

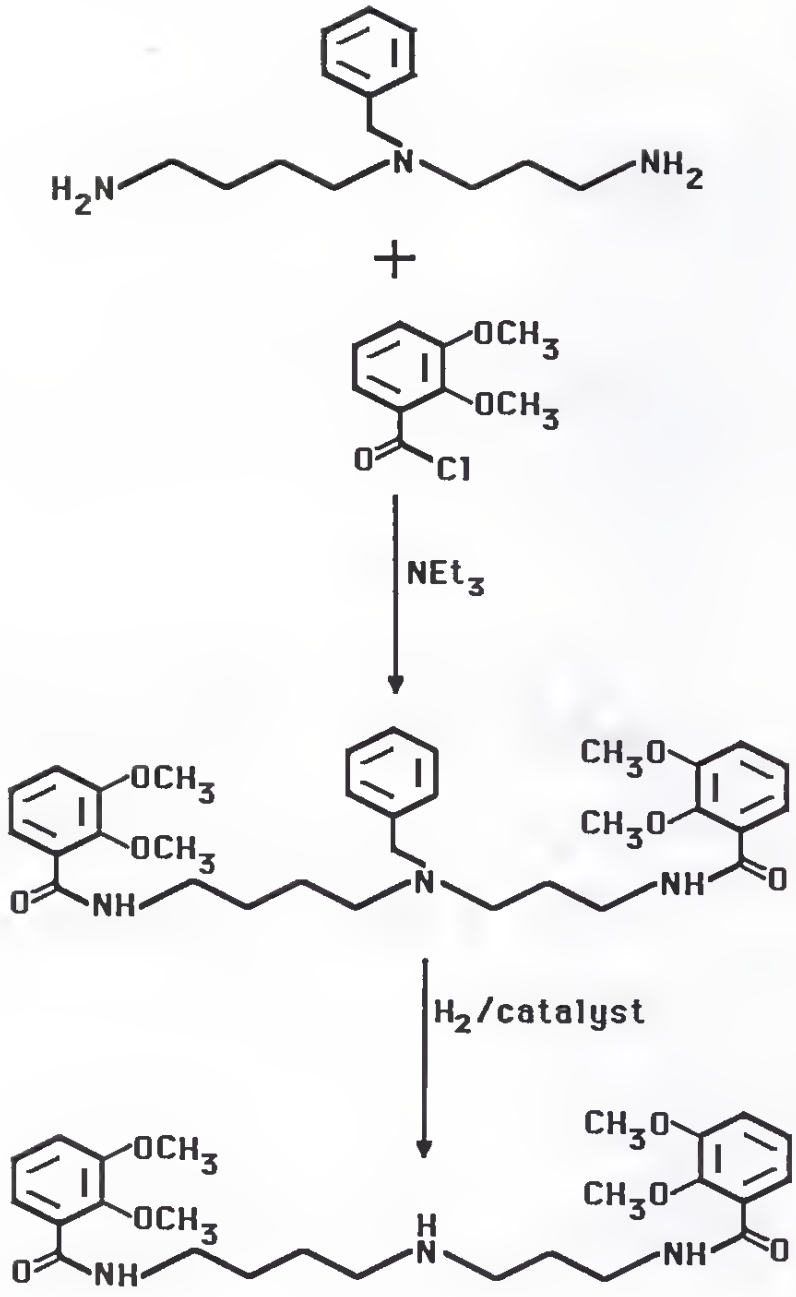


FIGURE 12
Synthesis for Tetramethoxy Precursors.

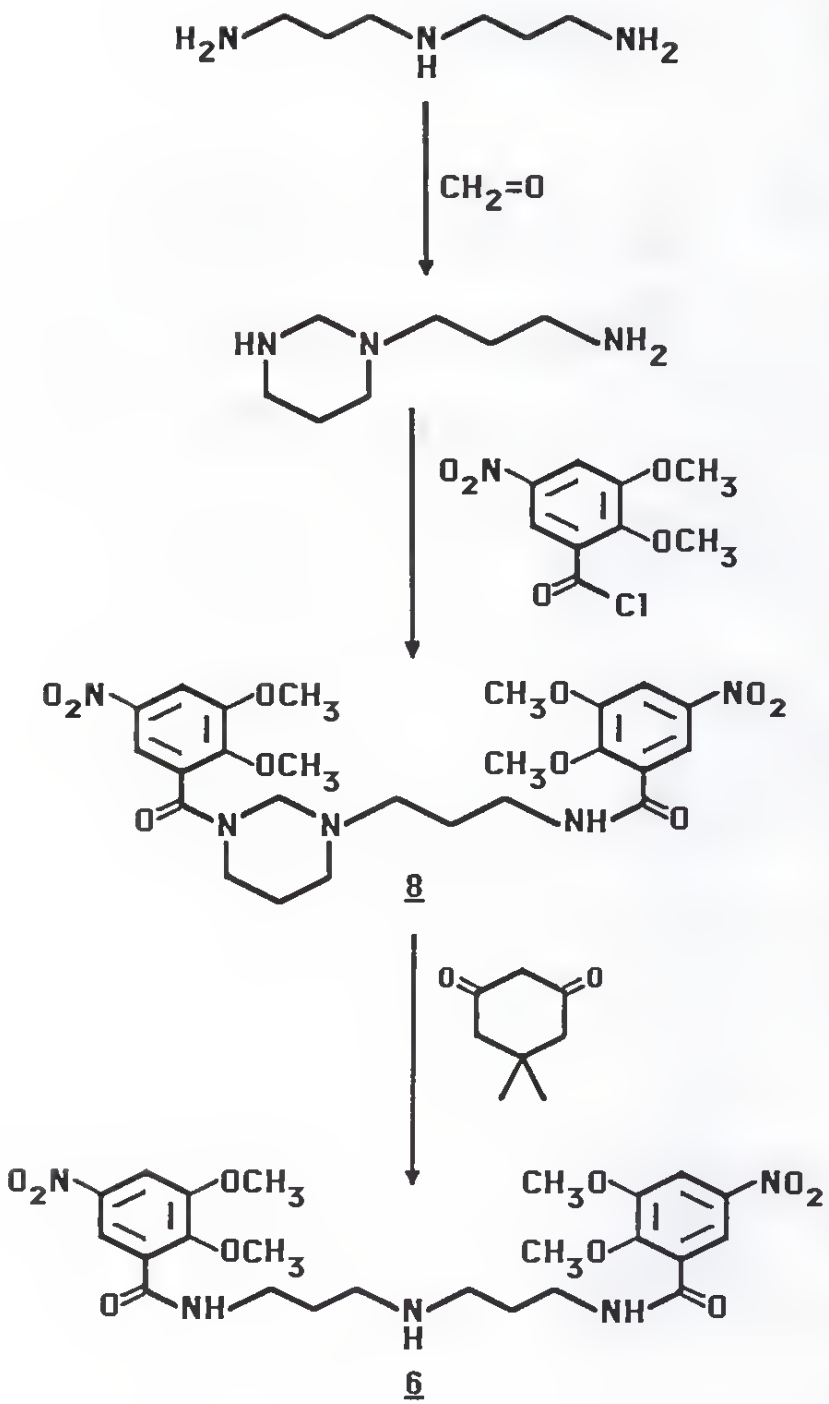


FIGURE 13
Synthesis for Bis Nitro Tetramethoxy Precursor.

synthesis will also proceed smoothly with nitro derivatives. Thus, one should be able to synthesize a bis nitro H-shaped ligand precursor in which two catechols are nitro-derivatized and two are not. Unfortunately, reaction of boron tribromide with 5a yielded a product which was not iron positive (52), indicating that free catechols are not present in the product. Trimethyl silyl halides were no more effective at freeing the catechols in this compound. This was very surprising in light of the fact that N,N-diethyl(2,3-dimethoxy-5-nitro)-benzamide deprotected in the usual manner, and that 6 was deprotected with facility as well.

N-(3-amino-1-propyl)hexahydropyrimidine (APHHP) has proven to be a very useful reagent to us for organic synthesis. In the preceding synthesis, the fact that the central amine of norspermidine is now tertiary has been exploited. Methods were also developed in which the difference between the primary and secondary amine is used to our advantage. If one were to add a single equivalent of an acid chloride to APHHP, the products would include primary-acylated, secondary-acylated, and bis-acylated hexahydropyrimidine, an undesirable result. By using an N-hydroxysuccinimide ester rather than an acid chloride, one can selectively acylate only the primary amine in near quantitative yield. Although other researchers have used hindered acyl transfer reagents or protecting groups to achieve the same end, these reagents are not nearly as selective (53, 54, 55). Once isolated, this mono-acylated product can react with a different acyl chloride, followed by deprotection with dimedone, to yield an asymmetrically substituted polyamine. Alternatively, the monosubstituted hexahydropyrimidine can be deprotected with dimedone, followed by reaction with a different N-hydroxysuccinimide ester to yield the identical product.

In order to demonstrate the usefulness of this method, 10 was prepared, figure 14. APHHP was acylated with (2,3-dimethoxy)benzoyl-N-hydroxysuccinimide, acylation occurring strictly on the primary amine. This product (9) was either reacted with 2,3-dimethoxy-5-nitro-benzoyl chloride followed by dimedone, or dimedone followed by (2,3-dimethoxy-5-nitro)-benzoyl-N-hydroxysuccinimide or (2,3-dimethoxy)benzoyl-N-hydroxysuccinimide, to yield 10 or

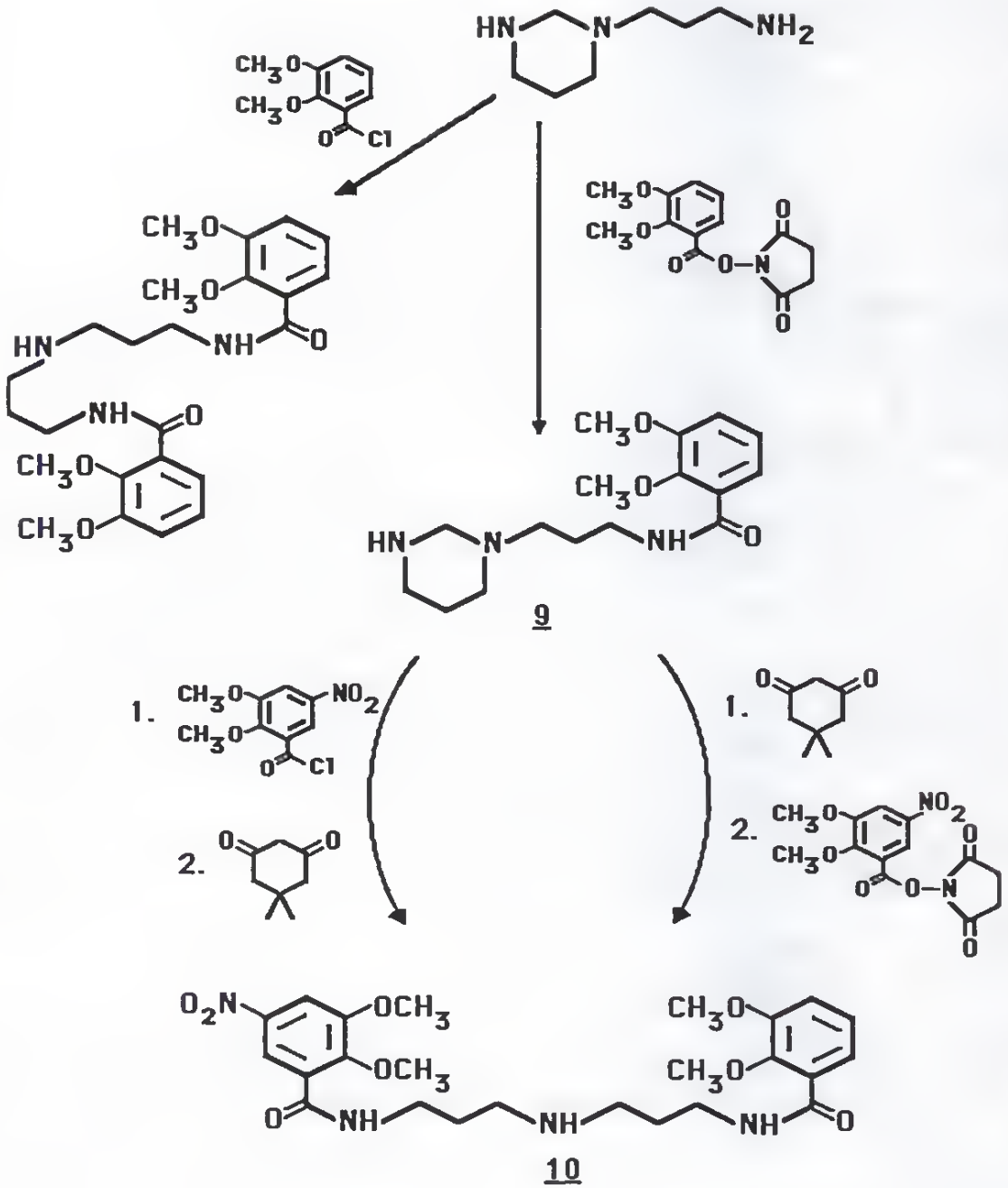


FIGURE 14
Synthesis for Mono Nitro Tetramethoxy Precursor.

N^1,N^7-bis-(2,3-dimethoxybenzoyl)norspermidine, respectively. To show that asymmetric synthesis will work with nitro derivatives, 10 was successfully reacted with 3a, figure 15, to yield the mono-nitro octamethoxy precursor. In this case reaction with boron tribromide proceeded with ease to yield 11a.

Resin-Bound Catecholamide Ligands

The catecholamide

The intention was to synthesize a catecholamide ligand with a chemical handle with which the molecule could be covalently bound to an inert resin matrix. The design of such a molecule required the integration of several factors: (1) in order for selectivity to be maintained it is crucial that the matrix-linked ligand retains the conformational mobility of the parent ligand, (2) the chemical handle must be capable of covalent binding with a resin material, (3) the placement of the handle into the molecule must be done in such a way that the geometry of the metal-ligand complex is undisturbed; i.e., the handle should be on the exterior of the chelate, (4) if the ligand were attatched directly to a polymer matrix, it is conceivable that the matrix would impose conformational restraints upon the ligand; i.e., the molecule would have restricted mobility, affecting its ability to sequester the metals of interest. Thus the handle should allow enough distance between the resin matrix and the chelator moiety so that the latter does not interfere with the mobility of the chelator and, more importantly, with metal chelation.

Compounds 1e and 1f with a chemical handle (13e and 13f) were synthesized by simply replacing glutaric acid with N-protected glutamic acid, figure 16. In this way a protected amine functionality is introduced into the backbone of the molecule, in a location which is far removed from the chelating sites. The t-butoxycarbonyl (BOC) group was chosen to protect the amine handle. This moiety is quickly and quantitatively removed by the action of triflouroacetic acid (TFA). A linear organic "string" in the form of N-BOC-8-aminocaprylic acid was attached to the amine handle to increase the distance between the ligand and the resin surface, figure 17. Once attached, a new amine functionality can be exposed by treatment with TFA to yield 15f.

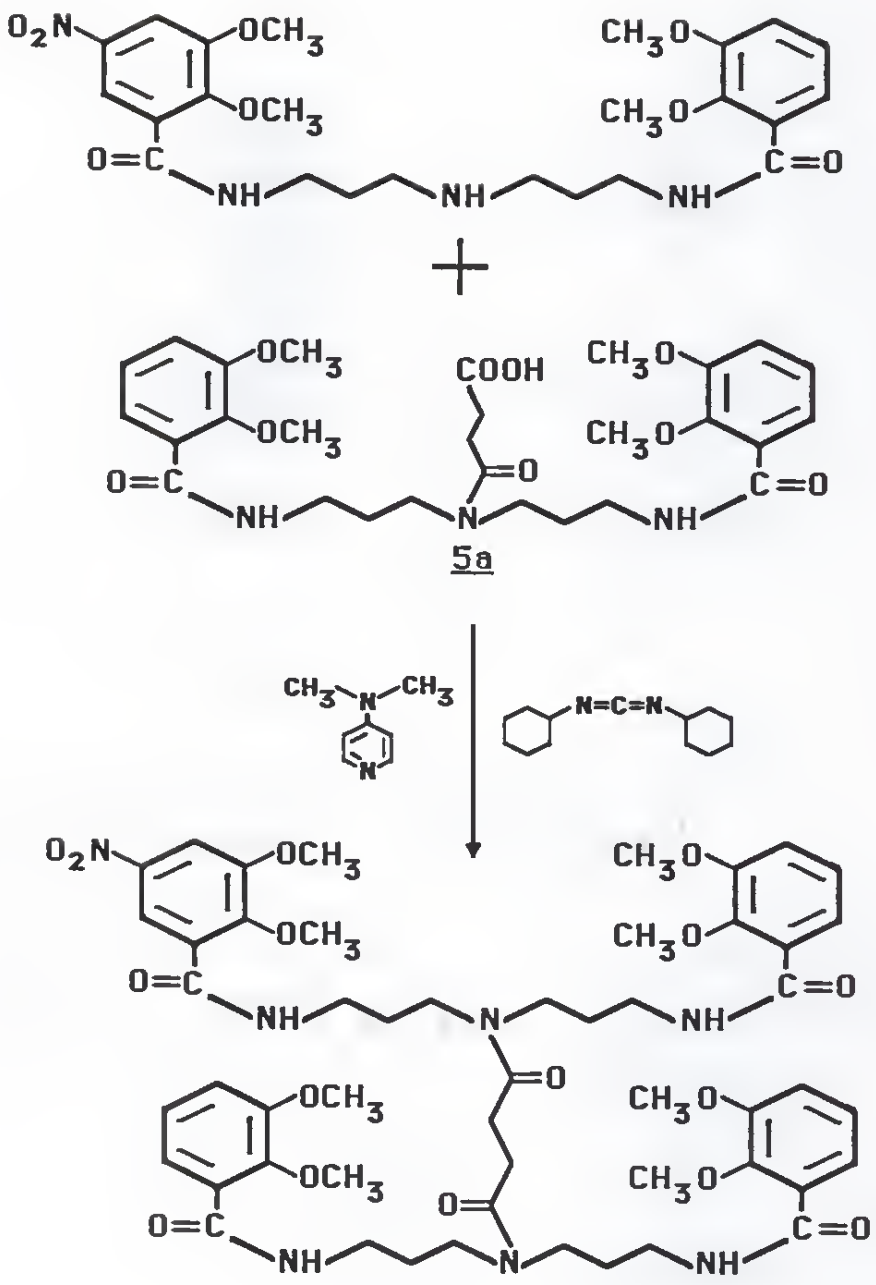


FIGURE 15
Synthesis for Mono Nitro Octamethoxy Precursor.

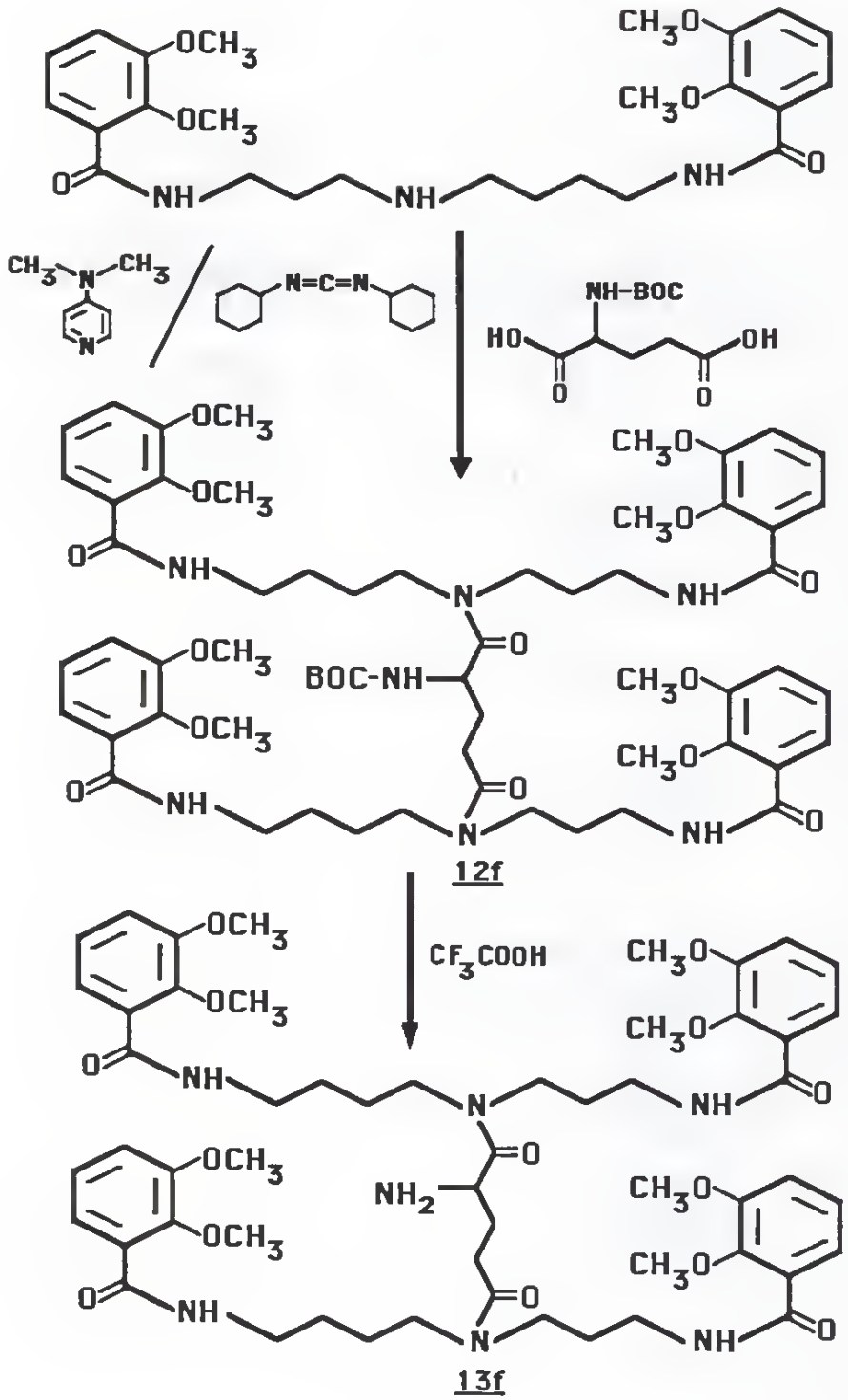


FIGURE 16
Synthesis for an Amino-H-Shaped Ligand.

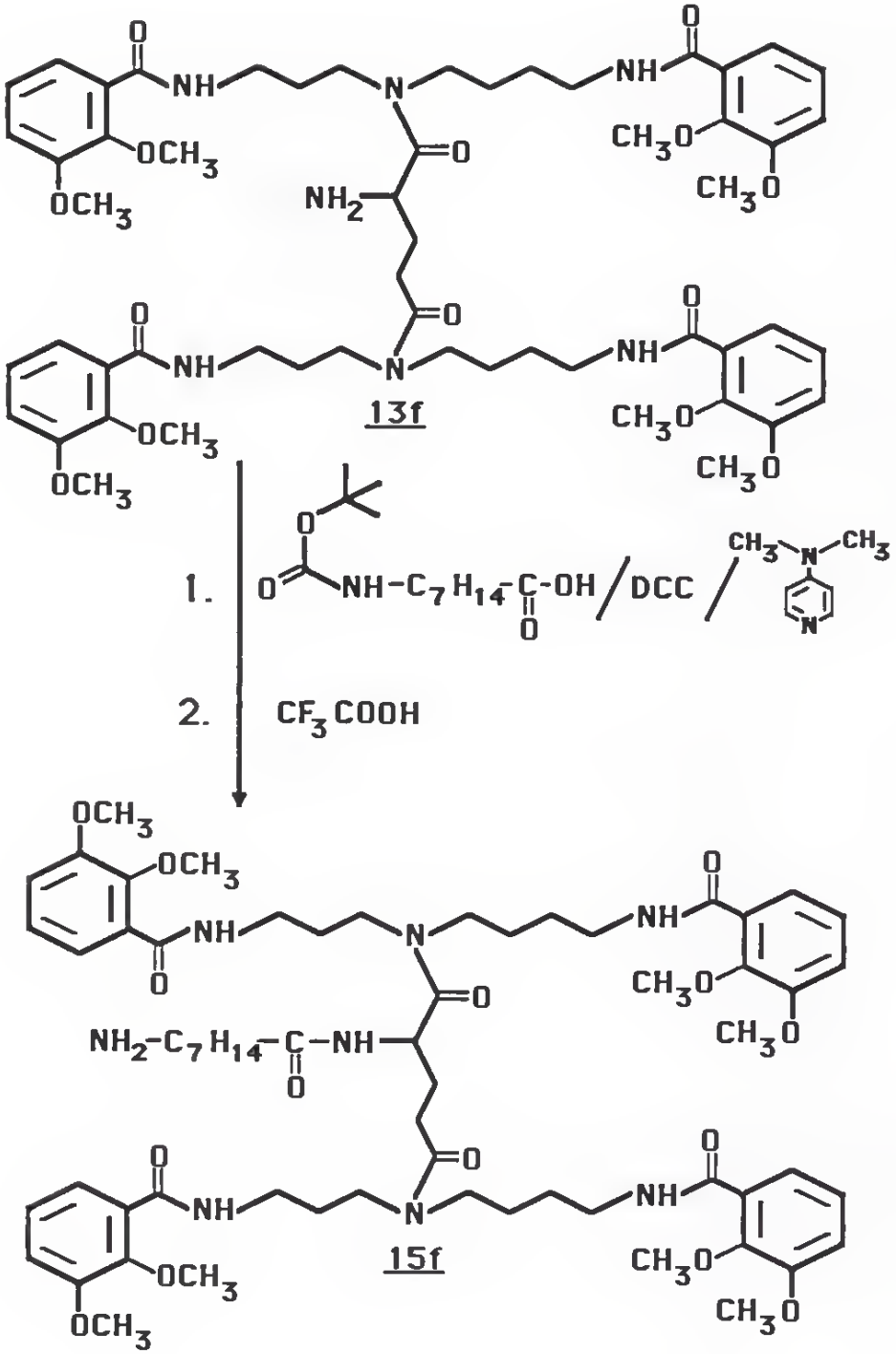


FIGURE 17
Attatchment of the Spacer Molecule.

Our first attempts to attach such a string involved 4-aminobutyric acid. The condensation went well but when exposed to TFA the product did not stain with ninhydrin, a reagent which qualitatively detects primary and secondary amines and amine salts. A possible explanation is shown in figure 18. However, when 8-aminocaprylic acid was used cyclization did not occur, yielding the desired product. An alternative synthetic pathway was also developed, figure 19. In this synthesis, N-BOC-8-aminocaprylyl-N-hydroxysuccinimate was reacted with glutamic acid, yielding the diacid derivatized with the organic string. This intermediate was reacted with two equivalents of N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine in high yield. In the previous synthesis, the H-shaped ligand was assembled prior to attachment of the organic spacer arm. This latter pathway renders the synthesis more cost effective because the synthesis is convergent rather than linear. Therefore, smaller building blocks, or intermediates, are used in the final steps.

The resin

It was decided that an amide linkage would be appropriate for coupling to the resin. Thus a carboxylic acid resin was sought. Merrifield's resin (56) is a commercially available resin composed of polyvinyl benzene monomers with a small percentage of the benzene moieties converted to their chloromethyl derivative, figure 20. This resin can be reacted with bicarbonate and dimethylsulfoxide to yield the aldehyde (57). Oxidation by dichromate in acetic and sulfuric acids yields the benzoic acid derivative 16 (58). Alternatively, reaction of the chloromethyl resin with malonic acid in the presence of pyridine and piperidine yields the cinnamic acid derivative 17. The advantage of having these two resins is that one can evaluate the effects of changing the length of the connecting string. Another resin, Amberlite IRP-64, is made from acrylic acid monomers, and already exists as a carboxylic acid (18). This resin was also used. These three resins were converted with thionyl chloride to their acid chlorides (59, 60) and reacted with the methylated amino-ligand 15f, yielding 19f, 20f and 21f, respectively. The degree to which the ligand coupled was determined by weighing the resin before and after exposure to the ligand.

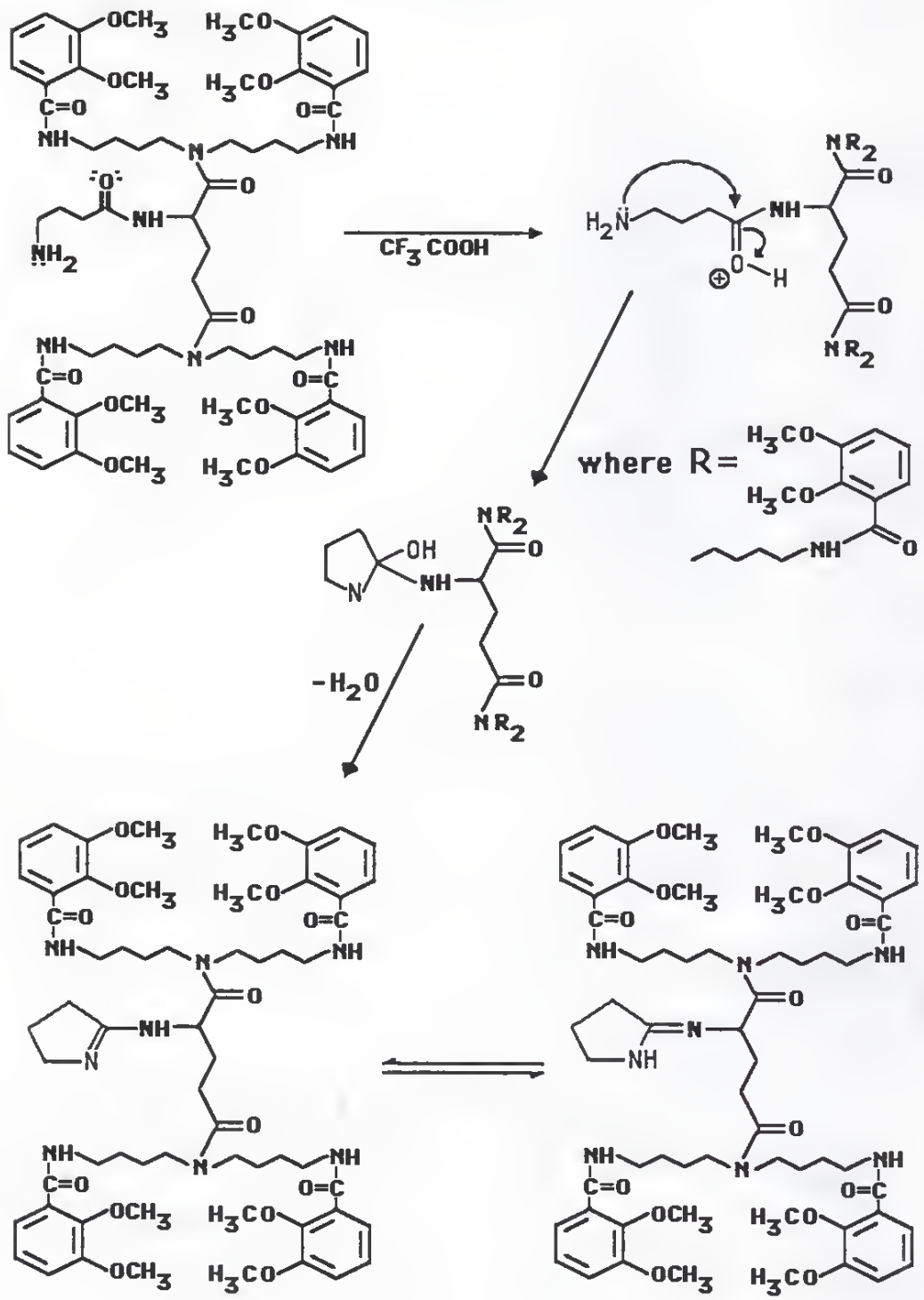


FIGURE 18
Cyclization Mechanism in Triflouroacetic Acid.

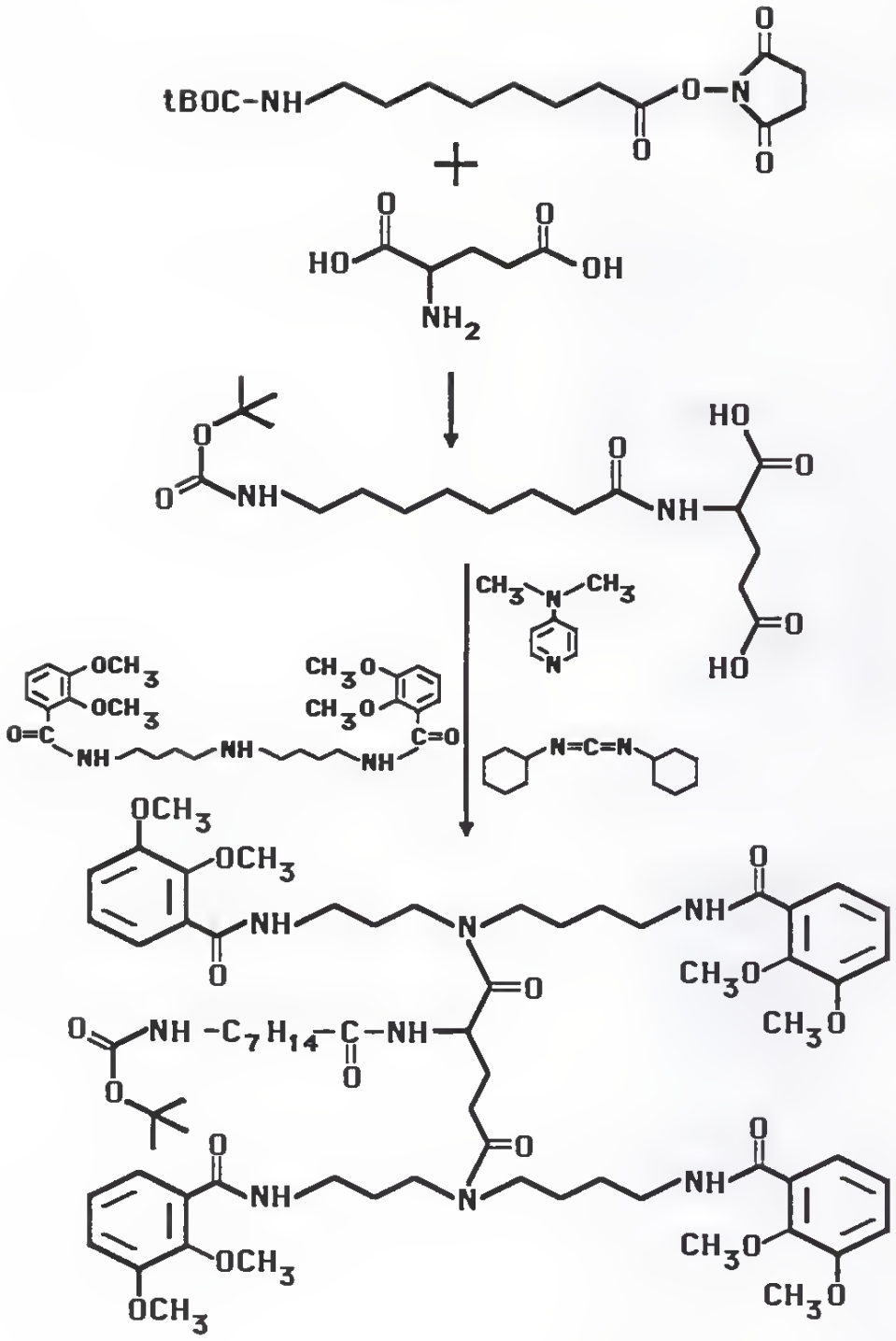


FIGURE 19
Alternate Synthesis for Amino-H-Shaped Ligand with Spacer Molecule Attatched.

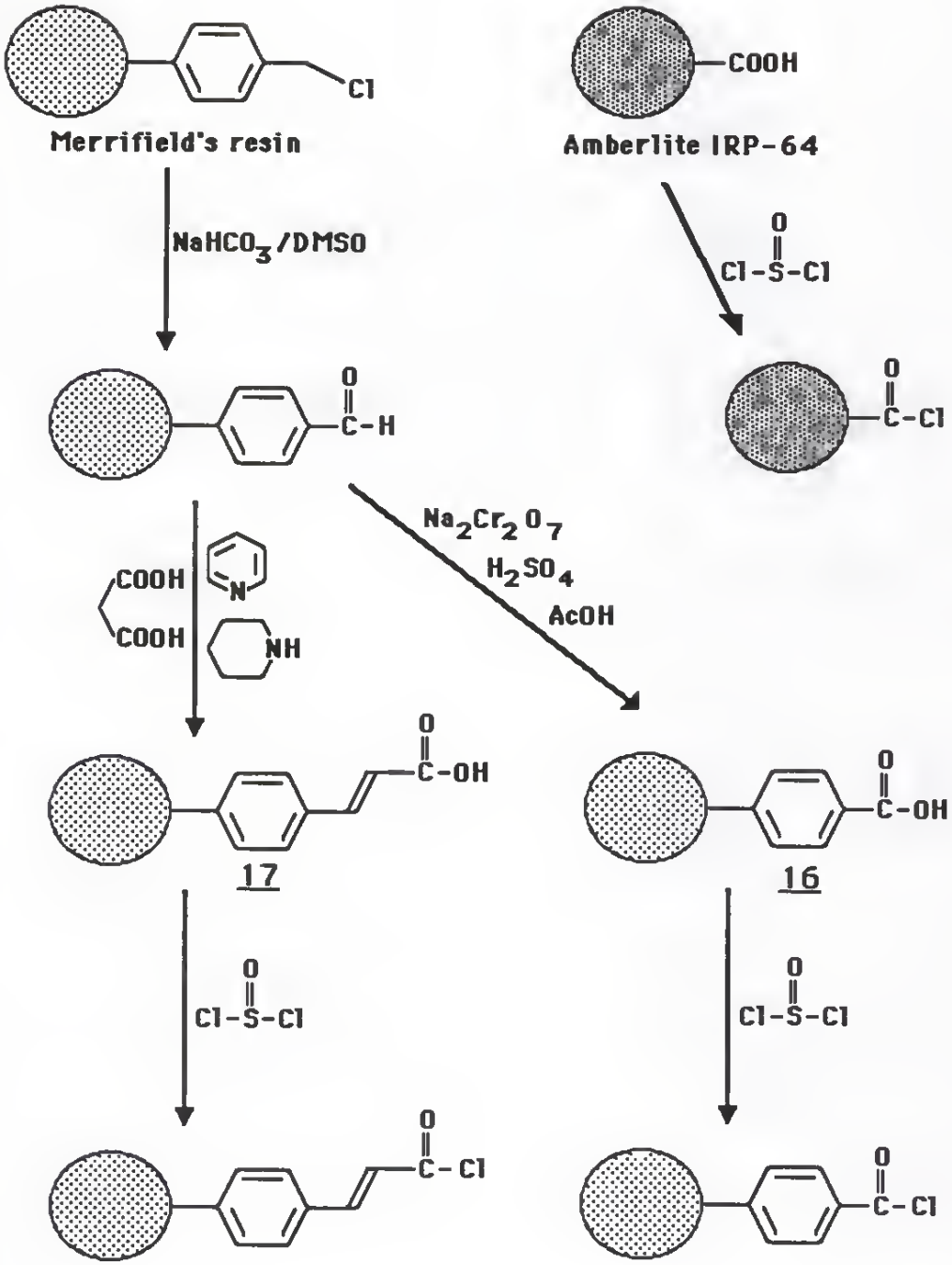


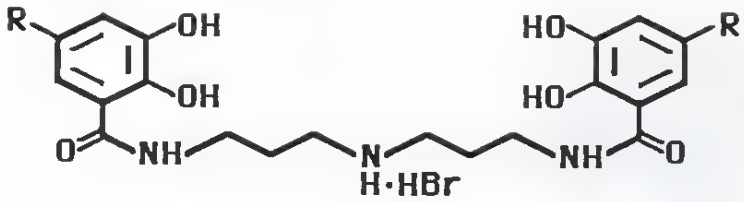
FIGURE 20
Synthesis for Acid Chloride Derivatives of Commercially Available Resins.

Reaction with boron tribromide gave the free catecholamide resins 22f, 23f, and 24f, as indicated by a positive reaction with ferric chloride solution (52).

Lastly, CH-Sepharose-4B activated resin was obtained. This resin was ideal for ligand immobilization. The matrix is composed of a polysaccharide backbone which has been derivatized with hexanoic acid present as the N-hydroxysuccinimide ester. Since this ester will react selectively with amines in the presence of alchohols, the methyl protecting groups were removed prior to coupling of the ligand to the resin material. Therefore the resin need not be exposed to the harsh chemical reaction conditions necessary for demethylation. This was not the case with the acid chloride resins described. Exposing 13f to boron tribromide gave a high yield of the desired "catecholamine" 25, figure 21. When 25 was coupled to the resin, giving 26, a spacer of six carbon units is inherently inserted between the ligand and the polymer surface, allowing for free motion of the molecule. Coupling was performed in 50 percent pH 7.2 phosphate buffer: 50 percent ethanol to ensure dissolution of the catecholamide. In addition, the resin was washed with a large volume of ethanolic buffer after the reaction was quenched to ensure removal of any uncoupled ligand. A final wash was performed with 50 percent pH 4.0 acetate buffer: 50 percent ethanol to protonate all of the catechoyl functionalities. The last wash was concentrated and reacted with $FeCl_3$. No color change was observed, indicating that no free catechol was present in this last wash. It was gravimetrically determined that ligand coupling was quantitative, by the weight difference between the control resin and the catecholamide resin. When a small amount of this resin (26) was placed in $FeCl_3$ solution, a dark purple color developed, indicating the presence of the catechoyl functionality.

Ligand Stoichiometry: Job's Plots

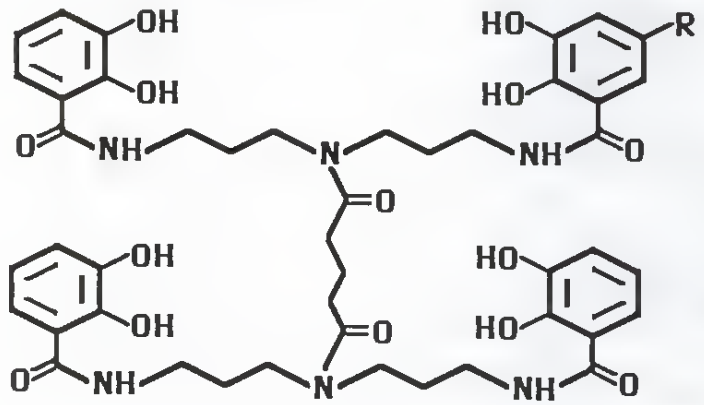
Before ligand binding constants could be calculated, it was necessary to measure the metal-ligand ratio for all metals and ligands involved in the determination. Thus, Job's plots (61) were performed in an ammonia buffer at pH 9.2 with copper for ligands 1a, 27, and Cpd II (N^1,N^8-bis[2,3- dihydroxybenzoyl]spermidine), figure 22, at a total concentration of metal and



27 $R = NO_2$

Cpd II $R = H$

M	Stoichiometry (L:M)
Th^{4+}	1:1
Cu^{2+}	1:1



11a $R = NO_2$

1a $R = H$

M	Stoichiometry (L:M)
Th^{4+}	1:2
Cu^{2+}	1:2

FIGURE 22
Results of Job's Plots with Catecholamide Ligands.

ligand of 33 μM. The results indicate that Compound II and 27 form 1:1 metal-ligand complexes with copper and 1a forms a 2:1 copper complex. These results are consistent with McGovern's findings for Compound II (62, 63). In addition, it is reasonable to expect 2:1 stoichiometry for 1a and copper, since 1a is essentially two Compound II molecules linked together.

In the case of copper, spectral changes upon metal binding are small, but are large enough for this type of measurement. With thorium, however, the spectral changes could not be used for Job's plot measurements. Therefore, the mono nitro H-shaped ligand 11a, figure 22, was used in place of ligand 1a. In order to show that using 11a would be a true reflection of what happens when 1a is used, a Job's plot was generated for 11a and copper. If the nitro group caused a significant change in the ability of the catechol to bind a metal, the Job's plot would not indicate 2:1 stoichiometry. For example, if the nitro-containing catechol binds copper more effectively, the first equivalent of copper would associate primarily with this group. This phenomenon would be reflected in the Job's plot as 1:1 stoiciometry, since the second equivalent of copper would not affect the nitro-containing catechol, which is already associated with the first equivalent of metal.

Conversely, if the nitro-containing catechol binds copper less effectively, the first equivalent of copper would not affect the visible spectrum since the metal would not associate with the nitro-containing catechol. Neither of these effects were observed, indicating that 11a does indeed accurately reflect the chelation chemistry of 1a.

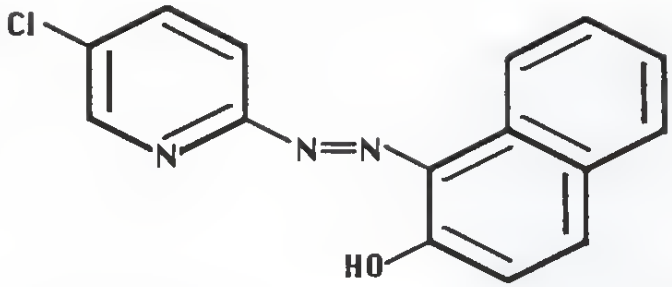
Thus, compunds 27 and 11a were used as a measure of the stoichiometry of the thorium complexes of Cpd II and 1a, respectively. For compound 1a another experiment was performed to insure the accuracy of using 11a in its place. A Job's plot was performed with 1a and thorium with eriochrome black T (EBT) present. It has already been shown that EBT does not displace thorium from H-shaped ligands under these conditions. Therefore, EBT can be used to complex any thorium which is not bound to 1a. All of these experiments yielded the same information: Cpd II and 27 form 1:1 metal-ligand complexes with thorium, and 1a and 11a form

2:1 complexes with thorium. This was somewhat surprising in light of the 1:1 stoichiometry observed with hexadentate catecholamide chelators and iron. This stoichiometry is most likely due to the ability of hydroxide to compete with 1a for the coordination sites of thorium at the experimental pH; i.e., ThO_2 is the specie bound. These results are summarized in figure 22.

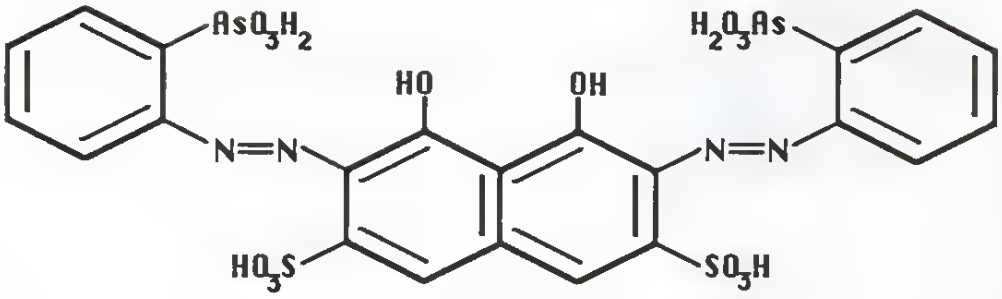
Ligand Precipitation Techniques: Removal of Actinides from Water

Several preliminary findings indicated that precipitation techniques would be a very effective means ot decontaminating radioactive solutions. In earlier studies on hexacoordinate catecholamide chelators, it was observed that neither the chelator nor the iron chelate could be dialyzed. Both species were held up on the dialysis membrane (64), suggesting that these chelators could be bound by a filtration membrane. Additionally, when a millimolar aqueous solution of H-shaped ligand was prepared at pH 10.0 and the pH was lowered below 8.5, precipitation occurred. This observation recurred when an equivalent of thorium(IV) or plutonium(IV) was present, implying that complex formation does not interfere with the process of precipitation.

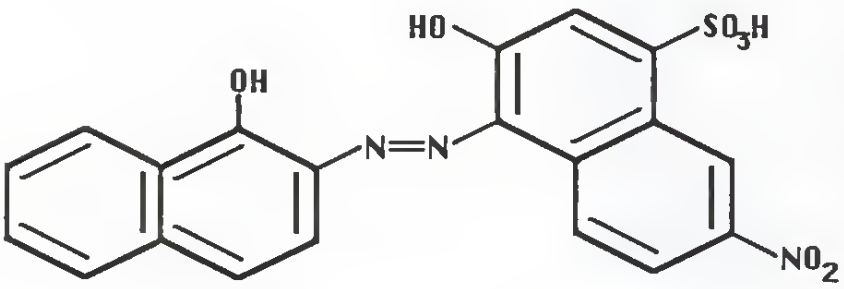
Several filtration techniques were developed to utilize the pH-dependent solubility properties ot H-shaped ligands for decontamination. In the tirst of these, it was determined that a metal-ligand complex could be removed from solution by filtration. A solution of thorium(IV) chloride buffered at pH 9 was added to several tubes. To these were added one equivalent ot an octacoordinate ligand in a small volume of methanol. Controls received only methanol. After neutralization, samples were filtered and the filtrates analyzed tor thorium. In order to have a benchmark for the data, the ligand 3,4,3-LICAM was synthesized and evaluated along with H-shaped ligands. Arsenazo III (ARS, figure 23) was used to colorimetrically detect the thorium(IV) remaining in solution (65). Absorbance maxima for the thorium-ARS complex were observed at 615 nm and 666 nm. The 666 nm peak was used tor the quantitative determination ot thorium(IV) because there was less absorbance by free ARS at this wavelength than at 615 nm. It was tound that 96.39-99.97 percent of the actinide could be removed in this



Cl-PAN



Arsenazo III



Eriochrome Black T

FIGURE 23
Colorimetric Reagents used for the Quantitative Detection of Metals.

way, first column of table 1. Note that 3,4,3-LICAM did not perform as well as H-shaped ligands in this experiment, removing 88.9 percent of the actinide. Controls without a ligand showed no removal of actinide.

In a series of experiments conducted in a joint effort between Rocky Flats and the University of Florida it was demonstrated that plutonium could be removed from aqueous solution by the same precipitation technique (J. D. Navratil, private communication), table 2. In practice, this means that if contaminated water is adjusted to high pH and a ligand is added, the contamination can be concentrated onto a filter by neutralization prior to filtration.

One might argue that a portion of the actinide, due to the nature of the experiment, was simply trapped by precipitation of the ligand in a non-specific way. If this were the case, the ligand would be able to bind more metal than the amount dictated by the ligand-metal complex. It was shown that this is not the case. When the ligand precipitation experiment was repeated with excess thorium, the ligand did not remove more than the stoichiometric amount of metal, indicating that non-specific precipitation did not occur.

The next experiments determined that H-shaped ligands can bind thorium(IV) when the metal is introduced into an acidic solution containing the precipitated ligand and, conversely, that the ligands can effectively precipitate thorium when introduced into an acidic solution containing the actinide. This renders it unnecessary to adjust the solution pH prior to addition of the chelate, an important financial consideration.

First, ligand was precipitated from a basic solution by acidification. One equivalent of thorium(IV) was then added. The suspension was stirred for several minutes, filtered, and the effluent assayed for thorium. Then, an acidic thorium solution was prepared. One equivalent of a ligand was added as a methanolic solution. The suspension was stirred for several minutes, filtered, and the effluent assayed for thorium. These methods proved very effective, removing 93.04-99.91 percent of the metal, table 3.

TABLE 1
Results of Thorium Precipitation Experiments With Catecholamide Ligands.

Compound	Percent Thorium Removal[a]		
	Competing Metals Absent	Competing Metals Present[c]	Percent Difference
1a	96.90 ± 1.42 (5)[b]	85.86 ± 4.19 (6)	11.39
1b	99.76 ± 0.35 (6)	89.67 ± 4.19 (5)	10.11
1c	97.77 ± 2.72 (6)	88.49 ± 2.86 (6)	9.49
1d	96.75 ± 0.95 (6)	85.58 ± 4.95 (6)	11.55
1e	99.38 ± 1.29 (6)	93.76 ± 4.40 (6)	5.66
1f	96.39 ± 1.06 (6)	84.32 ± 4.33 (6)	12.52
1h	99.74 ± 0.45 (6)	89.54 ± 2.31 (6)	10.23
1j	99.59 ± 0.55 (4)	84.96 ± 3.93 (6)	14.69
1k	99.97 ± 0.05 (5)	85.38 ± 5.87 (6)	14.59
LICAM	88.90 ± 3.40 (5)	71.05 ± 7.53 (6)	20.08

[a] $[Th]_{initial}=3.24 \times 10^{-5}$ M, $[ligand]_{initial}=3.3 \times 10^{-5}$ M.
[b] The number in parentheses is the number of experiments performed.
[c] Competing metals used were Fe(III), Ca(II), Mg(II), Mn(II), Zn(II), and Hg(II). One equivalent of each metal was used.

TABLE 2
Results of Plutonium Precipitation Experiments With Catecholamide Ligands.

Compound	% Plutonium Removal	
	Without Iron	With Iron
1a	68 ± 3	59 ± 5
1b	90 ± 6	86 ± 6
1d	63 ± 11	58 ± 6
1e	55 ± 1	47 ± 4
1f	92 ± 10	—
1j	88 ± 9	—
1k	66 ± 11	54 ± 6

$[Pu]_{initial} = [ligand]_{initial} = 1 \times 10^{-5}$ M.

TABLE 3
Results of Catecholamide Pre-Precipitation Experiments with Thorium.

Compound	Percent Thorium Removal: Ligand Added to an Acidic Metal Solution		Metal Added to an Acidic Ligand Suspension	
1a	99.91 ± 0.16	(4)[a]	96.90 ± 3.41	(6)
1b	98.21 ± 3.15	(5)	99.16 ± 0.82	(6)
1c	99.83 ± 0.19	(6)	98.90 ± 1.66	(6)
1d	99.54 ± 0.79	(5)	93.04 ± 6.98	(6)
1e	95.60 ± 6.21	(6)	99.08 ± 1.44	(6)
1f	99.40 ± 0.63	(6)	98.93 ± 0.51	(5)
1h	99.82 ± 0.31	(4)	99.37 ± 0.46	(6)
1j	94.58 ± 4.18	(5)	99.35 ± 0.57	(6)
1k	94.31 ± 7.20	(5)	98.40 ± 1.81	(5)
LICAM	97.64 ± 2.00	(5)	97.95 ± 1.02	(6)

[a]The number in parentheses is the number of experiments performed. $[Th]_{initial}=3.24 \times 10^{-5}$ M, $[ligand]_{initial}=3.3 \times 10^{-5}$ M.

A property which is requisite for the H-shaped ligands in order to be practical for decontamination purposes is their ability to sequester actinides in the presence of other metal ions. Thus, experiments were designed to determine whether other polyvalent cations could compete with actinides for the ligand binding site. Cations were selected because of their +2 and +3 oxidation state, their relative abundance and importance in biological systems, and their known ability to compete with actinides for binding with other chelating agents. These experiments involved repeating the procedures outlined above with an equivalent of each competing metal present before introduction of the ligand. Control experiments without thorium(IV) were also performed to determine if the other cations could form a complex with ARS, causing a colorimetric reaction. Very little interference was detected, which suggests that the cations should not affect formation of the thorium-ARS complex to any significant extent. Although substantial thorium removal was still observed--93.76 to 84.32 percent--the competing metals did have some effect, and some ligands were affected to a greater extent than others, table 1. For example, 1e demonstrates the most selectivity while 1j and 1l are the least selective H-shaped ligands. Once again, H-shaped ligands removed much more thorium than the 71.05 percent removed by 3,4,3-LICAM. In addition to removing less metal, 3,4,3-LICAM is the least selective catecholamide, demonstrating a 20.08 percent decrease in thorium removal.

At Rocky Flats, the ability of iron (III) to compete with plutonium for the ligand was measured, table 2. It was found that iron exerted a small effect on plutonium binding, a result consistent with our observations. Since iron forms a very stable chelate with the catechoyl moiety, it was somewhat surprising to us that iron did not compete more effectively for the octacoordinate ligand. These results indicate that it is reasonable to expect octacoordinate catecholamide ligands to experience little interference from biological iron when searching for absorbed actinides.

Insoluble Chelators: Removal of Actinides from Water and Blood Plasma

Although very effective as chelators, H-shaped ligands have some limitations for applicability. For example, a chelator taken by mouth to prevent GI absorbtion would be useless if absorbed from the gut. Additionally, the filtration technique is not applicable to basic solutions, where the ligands are very soluble. A soluble ligand would also be inappropriate for dialysis. Therefore we wanted to modify the ligand into an insoluble form, to overcome the types of problems just mentioned.

XAD-4-Sorbed Ligands

The Rocky Flats group demonstrated that when our H-shaped ligands are adsorbed onto XAD-4, a macroreticular resin, and contacted with aqueous plutonium-containing solutions, plutonium(IV) adsorbs very effectively onto the resin (66). In these early studies, excessive amounts of ligand were coated onto the resin, resulting in a situation where a great deal of the ligand was not available to sequester plutonium from solution; i.e., ligand exposure on the resin was minimal. Presumably the ligand molecules stacked on top of one another rather than forming a monolayer. In spite of this, remarkable decontamination factors (Df's) were observed, values in the hundreds. The Df is defined as the moles of metal bound per gram of ligand divided by the moles of metal in solution per milliliter of solution. This value, of course, is difficult to use for comparison of various ligands because the molecular weight of the ligand is incorporated into this term. Therefore, it is more useful to measure the Kd for the system, defined as the moles of metal bound per mole of ligand divided by the moles of metal in solution per milliliter of solution. Df's are still presented to facilitate comparison to earlier work. In later experiments where the ligand loading had been optimized, Df's on the order of 10^7 were attained (67). Furthermore, the Rocky Flats group demonstrated that a significant amount of plutonium(IV) was removed from human plasma (up to 36 percent) when 10 mL were exposed to 250 mg of resin at 37°C overnight. This result indicates that resin-bound H-shaped ligands may be practical for dialysis. Their results are summarized in tables 4, 5, and 6.

TABLE 4
Kd's and Df's for Plutonium at a Ligand Loading of 10^{-6} moles ligand/0.25 g XAD-4.

Compound	Kd	Df
1a	2.69×10^{6}	1440
1b	7.48×10^{5}	1140
1c	9.57×10^{5}	1160
1d	5.30×10^{6}	1560
1e	2.25×10^{6}	1430
1f	2.12×10^{7}	2300
1h	3.75×10^{6}	1501
1j	6.60×10^{6}	1810
1k	3.69×10^{5}	1110
1l	4.40×10^{6}	1530
LICAM	4.81×10^{6}	1980

250 mg loaded resin was exposed to 10 mL solution containing 5×10^{-7}M plutonium. Samples were mixed overnight prior to measurement. Values have been corrected for plutonium removal by control resin.

TABLE 5
Kd's and Df's for Plutonium at a Ligand Loading of 10^{-7} moles ligand/0.25 g XAD-4.

Compound	Kd	Df
1a	2.88×10^7	14,600
1c	7.50×10^6	11,900
1d	1.15×10^8	10,200
1e	2.74×10^7	12,500
1f	4.50×10^7	11,500
1j	5.31×10^6	11,500
1k	3.95×10^6	11,200
1l	3.85×10^7	14,700
LICAM	2.03×10^7	16,100

250 mg loaded resin was exposed to 10 mL solution containing 5×10^{-5}M plutonium. Samples were mixed overnight prior to measurement. Values have been corrected for plutonium removal by control resin.

TABLE 6
Results of XAD-4 Experiments With Human Plasma.

Compound	Percent Plutonium Removal[a] A	B
1a	8 ± 6[b]	3 ± 19
1b	0 ± 6	0 ± 6
1c	21 ± 8	26 ± 10
1d	17 ± 3	0 ± 7
1e	28 ± 17	8 ± 18
1f	36 ± 8	9 ± 4
1h	n.a.	3 ± 4
1j	30 ± 13	6 ± 6
1k	22 ± 0	n.d.
1l	21 ± 8	0 ± 17

A = 10^{-3} moles ligand/0.25g XAD-4, B = 10^{-6} moles ligand/0.25g XAD-4.
[a]250 mg loaded resin was exposed to 10 mL plasma containing 4.18 x 10^{-7}M plutonium. Samples were mixed overnight prior to measurement.
[b]Values have been corrected for plutonium removal by control resin.

Covalently-Bound Ligands

IRP-64-bound catecholamides

The precipitation experiments previously described for free ligand were repeated using resin 24f in place of a free ligand, but without adjusting the pH to neutrality prior to filtration. Remember, pH adjustment was necessary to cause precipitation of the ligand-metal complex. In this case, the ligand is insoluble at every pH. Therefore, pH manipulation is unecessary. It was found that control resin--in this case the methylated catecholamide resin--removed as much thorium as the catecholamide resin. It was concluded that the polymer matrix was responsible for a large amount of metal binding. Unfortunately, many free carboxyl groups remain in the derivatized resin; only about ten percent reacted with a ligand. These free carboxyl groups are able to bind actinides, and therefore can interfere with attempts to measure metal binding due only to the bound ligand. Although these carboxyls appear to enhance thorium binding, this binding is non-specific. In order to measure the ability of the chelator, once bound to a polymer matrix, to bind thorium selectivly, binding due to the non-specific matrix must be either measured or prevented; we chose the latter approach.

Control resin was placed in pH 9.2 buffer with thorium and different concentrations of EDTA. It was found that an EDTA concentration of 150 mM was needed to prevent binding of thorium to the control resin, table 7. Unfortunately, this concentration is high enough to compete effectively under these conditions with the catecholamide for the metal, as indicated by reduced thorium removal by 24f at this concentration. It was concluded that a different resin should be used.

CH-Sepharose-4B-bound catecholamides

Next, resin 26 (figure 21) was tested. In this case 2.6 mM EDTA was enough to prevent the control resin from binding thorium when present at concentrations up to 0.64 μM, figure 24. In this case the control resin was reacted with ethanolamine, the reagent used to quench the excess reactive sites on the catecholamide resin. This concentration of EDTA did not affect

TABLE 7
Results of Experiments With IRP-64 Resin Derivative.

[EDTA]	Resin	% Removal	Hours	Kd
15 mM	—	0	3	2.22×10^3
	OMe	23.5		
	OH	71.1		
150 mM	—	0	6	2.11×10^3
	OMe	8.1		
	OH	47.8		
150 mM	—	0	24	5.21×10^3
	OMe	0		
	OH	65		

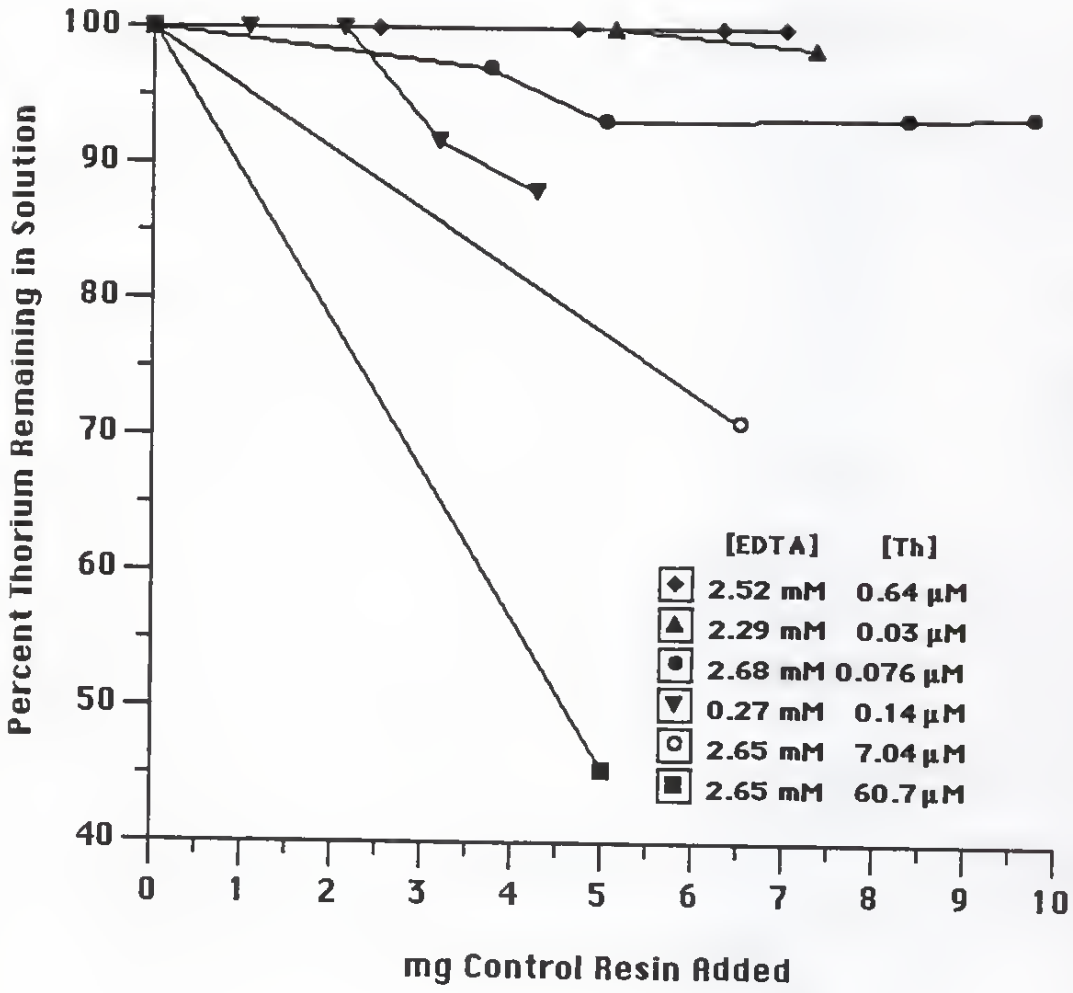


FIGURE 24
Determination of the Ability of EDTA to Prevent Adsorption of Thorium onto CH-Sepharose-4B Control Resin at pH 9.2 as a Function of the Milligrams of Resin Used.

thorium binding by the catecholamide portion of 26, figure 25. For these experiments, ^{230}thorium, a high energy α-emitter, was used, enabling very precise measurements of trace amounts ot metal to be made.

By adding ^{230}thorium to a solution ot ^{232}thorium, the concentration of metal can be increased while the number of counts added remains constant. When the concentration ot thorium was increased in this way trom 30 nM to 76 nM, it was found that control resin did bind some thorium, approximately six percent. When the thorium concentration was raised to 7.04 μM, more binding was observed; approximately 29 percent of the thorium in solution was non-specifically bound by control resin. In both cases, 26 was still able to remove more than 98 percent of the thorium in solution, figure 25. When competing metals were added, no significant difference in thorium removal by resin-bound catecholamides was observed, tigure 26. These data indicate that matrix-bound ligands represent an effective form of H-shaped ligands which could be applied to many facets ot decontamination.

Equilibrium Solution Chemistry: Thermodynamic Binding Constant Measurements

Eriochrome Black T Competition

Many techniques cannot be used to accurately determine very large metal-ligand binding constants due to the fact that the free metal in solution, or the free ligand in solution, is present at a concentration which is so low that it cannot be measured accurately. Theretore, it is essential to approximate this constant betore designing an experiment for an accurate determination. A spectrophotometric technique was developed to approximate the magnitude ot the thorium-ligand association constant. The colorimetric reagent eriochrome black T (EBT) was very well suited tor this approximation, tigure 23. EBT torms a very stable 1:1 complex with thorium(IV), the absolute binding constant being on the order ot 10^{27} M^{-1}. Its working pH ranges from 8 to 10, which is ideal for H-shaped ligand solubility (69). At its working pH EBT exhibits an absorbance maximun at 660nm while the thorium-EBT complex has an absorbance maximun at 570nm (70). A 10^{-5}M solution of EBT can quantitatively detect thorium(IV) to

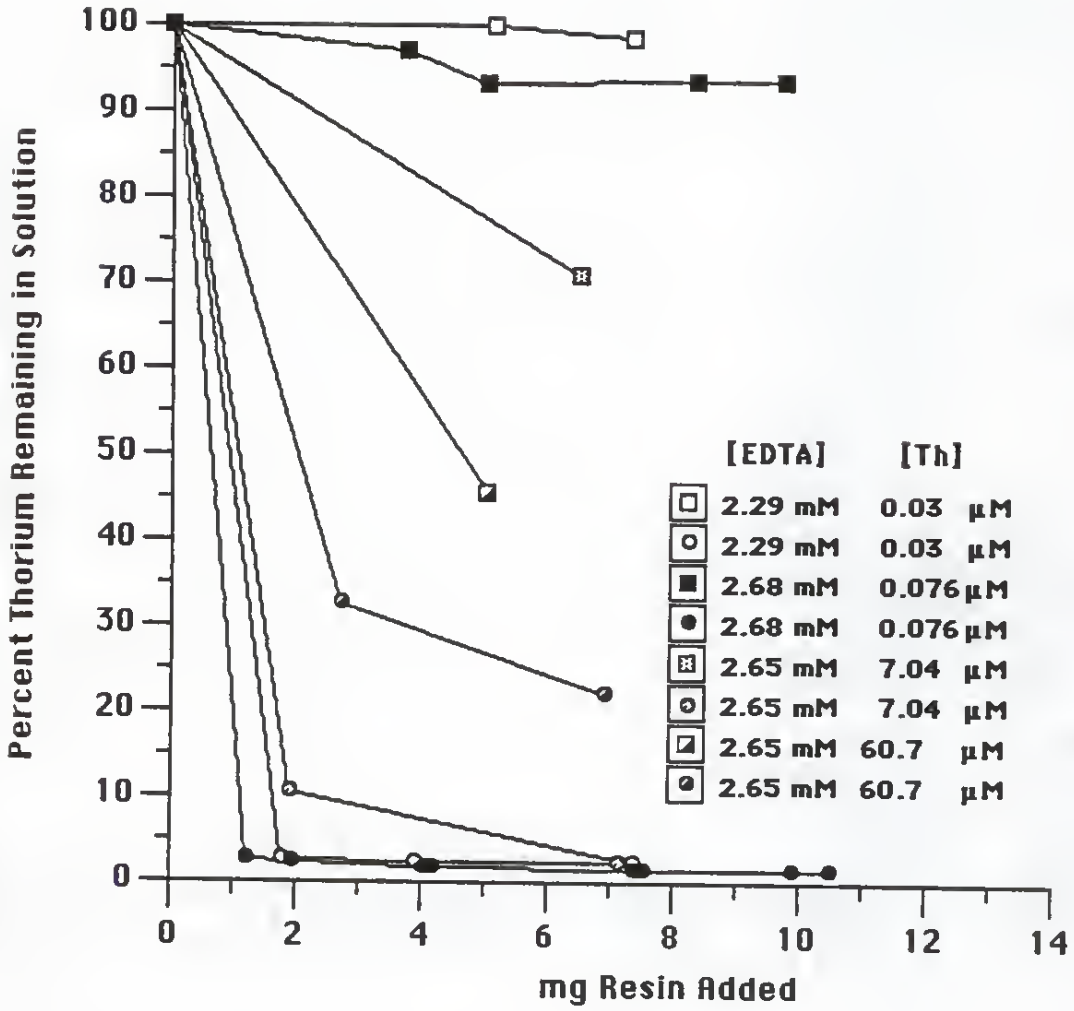


FIGURE 25
Determination of the Ability of 26 to Remove Thorium from Aqueous solution (pH 9.2).
Control Resin is Indicated by Squares and 26 is Indicated by Circles.

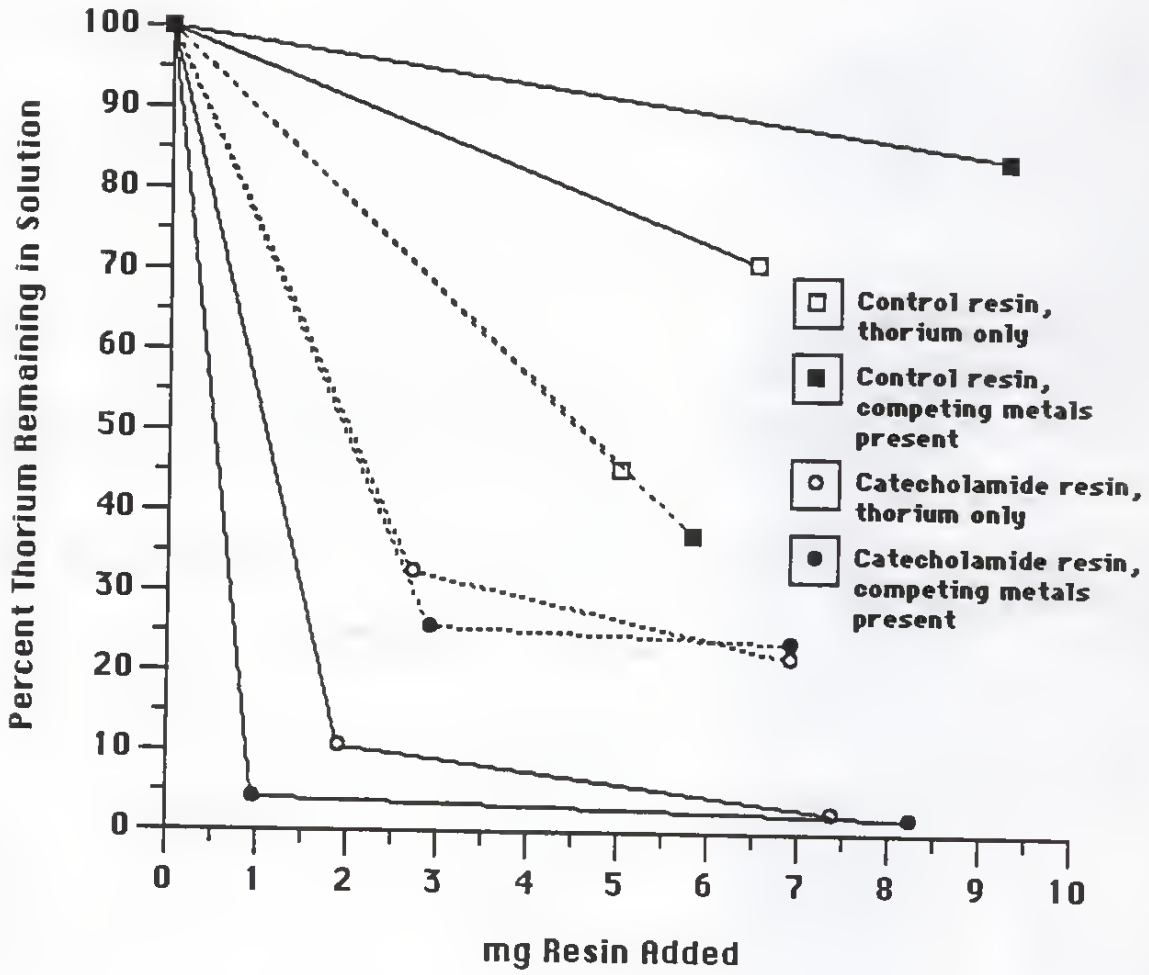


FIGURE 26
Determination of the Ability of Other Metals to Interfere with Removal of Thorium from Aqueous Solution (pH 9.2) by 26. Metals Used were Fe(III), Ca(II), Mg(II), Zn(II), Mn(II), and Hg(II). Solid Lines Represent 7.04 μM Metals, Dotted Lines Represent 60.4 μM Metals.

concentrations as low as 10^{-7}M (a 100:1 ratio of EBT to thorium) with a variance of one percent, figure 27.

To estimate the catecholamide-thorium(IV) association constant, a solution buffered at pH 9.2 was prepared, containing one equivalent of $ThCl_4$ and one equivalent of EBT. The solution showed a pink color, indicative of the thorium-EBT complex. Upon addition of one equivalent of a catecholamide, a color change, from pink to blue, was observed, indicating that the catechol displaced thorium from EBT, according to the equation

$$\underset{\text{pink}}{2\ \text{EBT-Th}} + \text{Cat} \rightleftharpoons \underset{\text{blue}}{\text{EBT}} + \text{Cat-Th}_2$$

The resultant absorbance spectrum was identical to that of the uncomplexed EBT dye, table 8. From these data it can be concluded that less than 0.1 percent of the total amount of thorium(IV) in the solution is associated with EBT. As a consequence of this "competition," the relative conditional binding constant of the catecholamide ligands relative to EBT must be on the order of 10^3 or greater.

Competition Studies with Nitro Derivatives of Catecholamide Ligands

In the previous experiment, the data could not be used for an accurate determination of the 1f-Th conditional metal binding constant because the fraction of thorium bound to EBT could not be measured. If one were to use ligands which shared the metal in such a way that the amount of metal associated with each ligand could be measured, accurate calculations of relative conditional binding constants could be made. If all proton stability constants, metal hydrolysis constants, metal-ligand complex stability constants, and metal-proton-ligand complex stability constants are known for one of the ligands, as well as the stoichiometry of the ligand-metal complex, then the conditional binding constant, defined by the equations

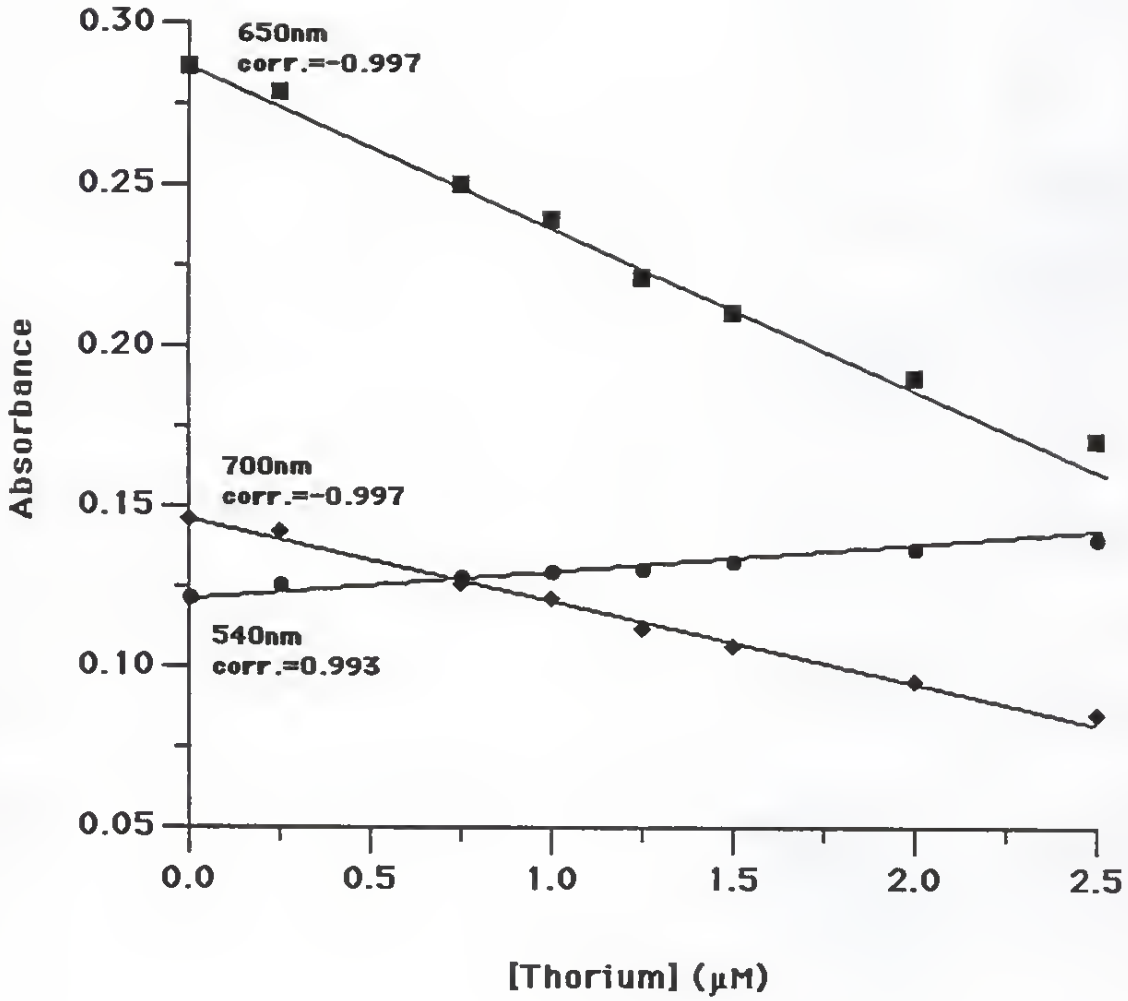


FIGURE 27
Standard Curve for $ThCl_4$ with EBT (10 µM).

TABLE 8
Color Reactions of Various Ligands with the Thorium-EBT Complex.

Compound	Color Reaction
Triethanolamine	-
EDTA	-
Salicylic acid	-
2,3-dihydroxybenzoic acid	-
3,4,3-LICAM	+
Compound 1a	+
Compound 1b	+
Compound 1d	+
Compound 1e	+
Compound 1f	+
Compound 1k	+

(+) indicates that the solution turned from pink to blue in 15 seconds or less upon addition of the ligand of interest. One equivalent each of EBT, thorium and ligand was used.

$$xM + nL \rightleftharpoons M_xL_n$$

$$\text{and} \quad K^* = \frac{(M_xL_n)}{(M)^x(L)^n}, \text{ where}$$

(M) = the total concentration of metal which is not complexed to the ligand.
(L) = the total concentration ligand which is not complexed to the metal.
(LM) = the total concentration of metal-ligand complex.
x,n = the stoichiometric amount of metal and ligand, respectively, in the complex,

can be calculated for that ligand-metal complex (71). If the relative conditional binding constant, defined as

$$K_{rel}^* = K_2^* / K_1^*, \text{ where}$$

K_1^* = the conditional binding constant for the first ligand-metal complex
K_2^* = the conditional binding constant for the second ligand-metal complex,

is measured, then the conditional binding constant for the second ligand can be calculated, since

$$K_2^* = K_{rel}^* \cdot K_1^*.$$

By repeating this two-ligand-one-metal competition, replacing one ligand with another, one can eventually arrive at the conditional binding constant for a ligand of interest. In an analogous fashion one can perform a one-ligand-two- metal competition experiment to arrive at the conditional stability constant for a particular metal or metals of interest.

In this particular case, it was hypothesized that another catechol would be a suitable candidate for competition with 1a. The problem was that during the process of compound characterization it was found that the spectral qualities of the H-shaped ligands--ultraviolet, visible, flourescent, etc. under a variety of conditions--did not change significantly enough upon thorium binding to be a useful analytical tool. Also, the changes of one catecholamide ligand upon metal binding could not be distinguished from changes in the other catecholamide. To avoid this complication a series of relative conditional binding constants were measured, utilizing nitrocatecholamides.

Besides increasing water solubility, N,N-diethyl(2,3-dihydroxy-5-nitro)-benzamide exhibited another potentially very useful property. During the course of titration, it was noted that the color of the solution containing this compound was pH-dependent. At pH lower than 7, the solution was yellow. At pH higher than 10, the solution was purple. At intermediate pH, the color ranged from yellow to orange to red to purple. We hoped to be able to put this "indicator" property of the nitro derivative to good use.

Since the effect of metal binding upon the UV-visible spectra of a compound is the same as loss of protons, it was our belief that, upon metal binding at the proper pH, the tetranitro derivative of an H-shaped ligand would show the same type of color changes exhibited by the anions of N,N-diethyl (2,3-dihydroxy-5-nitro)benzamide. As explained earlier, our attempts to synthesize such a derivative were unsuccessful. We were able, however, to synthesize a bis nitro derivative of Compound II, and hoped this compound would exhibit the same type of color changes as those seen with the anions of N,N-diethyl-(2,3-dihydroxy-5-nitro)-benzamide Thus, 6 was deprotected to yield N,N-bis(2,3-dihydroxy-5-nitrobenzoyl)-norspermidine (27), the idea being that upon metal binding spectral changes will occur in the visible range; in this range the parent catecholamide does not interfere with the absorption of the nitrocatecholamide. Thus if two catecholamide ligands are allowed to compete for one metal in solution, when one of the ligands, e.g. 27, contains a nitrocatechol and the other, e.g. N,N-bis(2,3-dihydroxybenzoyl)-spermidine (Compound II) or an H-shaped ligand, does not, the the amount of metal which is bound by the nitrocatecholamide can be quantitated by the changes in its absorption spectrum in the visible range.

Bergeron and McGovern (62, 63) have measured all of the equilibrium constants associated with formation of a metal complex with copper and Compound II. Since this type of data is not available for any thorium complex--due to the fact that the thorium hydrolysis constants are not known--we felt that we could use the data generated by McGovern, along with the copper-ammonia complex formation constants (72), to measure the copper-1a conditional

complex formation constant and eventually the thorium-1a conditional complex formation constant.

When copper was added to a constant concentration of the nitrocatecholamide 2Z, the absorbance at 400 nm was linear when plotted against the amount of copper added, figure 28. If we assume that all of the added copper is bound by the ligand, then a linear relationship exists between the analytical amount of copper and the concentration of ligand-copper complex, figure 29. If the same measurement is made with compound II or 1a present at the same concentration as 2Z, 2Z binds less copper than in the absence of the other catecholamide. By using the standard curve generated in the absence of a second catecholamide, the amount of copper bound to 2Z in the presence of compound II or 1a can be quantitated, and the percent of metal associated with the nitrocatecholamide can be calculated from the ratio of the slopes of the lines generated with and without a second catecholamide present, figure 29. If one knows the stoichiometric amount of copper in each complex of interest, the concentration of each metal complex can be calculated, leading to the relative conditional stability constant for the two ligands and copper.

In the cases of the Cpd II-copper complex and the 2Z-copper complex, x=n=1, and the relative conditional binding constant can be expanded to

$$K^{*}_{rel} = \frac{(M\text{-}L^{2})(L^{t})}{(M\text{-}L^{1})(L^{2})}$$

where

$M\text{-}L^{1}$ = the equilibrium concentration of the Cpd II-copper complex
$M\text{-}L^{2}$ = the equilibrium concentration of the 2Z-copper complex
L^{1} = the equilibrium concentration of Cpd II
L^{2} = the equilibrium concentration of 2Z.

Since excess ligand is present in all cases, free copper can be ignored, and

$$C_{M} = (M\text{-}L^{1}) + (M\text{-}L^{2})$$
$$C_{L}1 = (M\text{-}L^{1}) + (L^{1})$$
$$C_{L}2 = (M\text{-}L^{2}) + (L^{2}).$$

Since $C_{L}1 = C_{L}2 = 33.3\ \mu M$, let $C_{L}1 = C_{L}2 = C_{L}$. Then

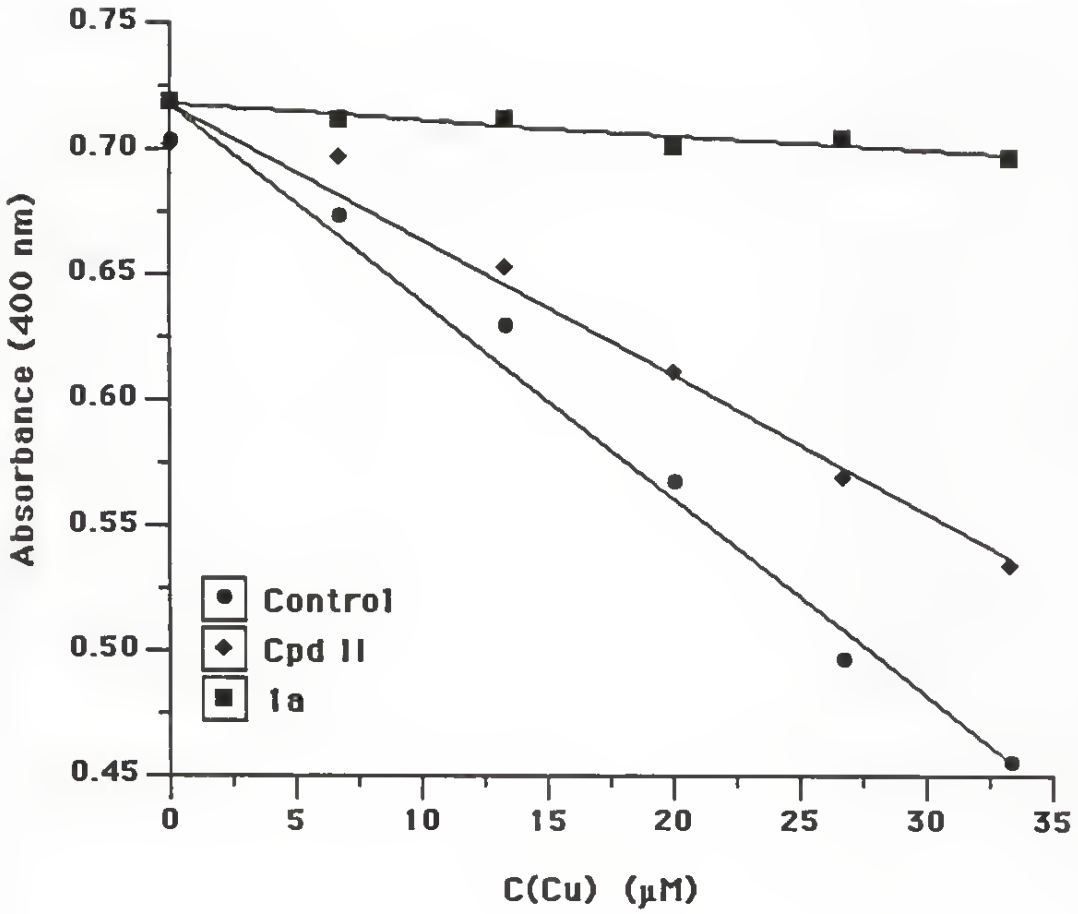


FIGURE 28
Results of Two-Ligand-One-Metal Competition Experiments where 27 is One Ligand and Copper is the Metal.

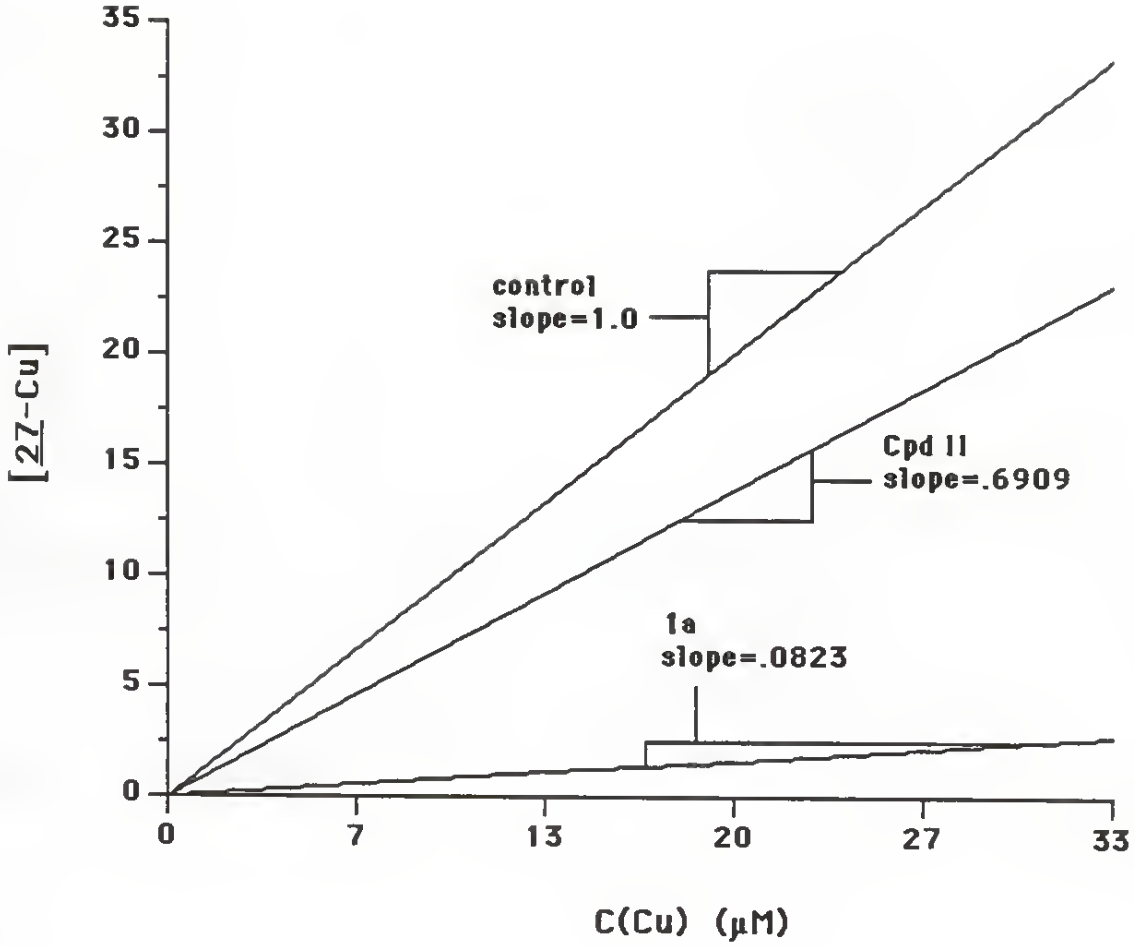


FIGURE 29
Plot of the Copper-27 Complex Versus the Amount of Copper Added. The Slope of the Line Represents the Fraction of Copper Associated with 27 at Equilibrium with an Equimolar Amount of Competing Catecholamide Present.

$$C_L = (L^1) + (M\text{-}L^1)$$
$$C_L = (L^2) + (M\text{-}L^2).$$

Rearranging these equations,

$$(L^1) = C_L - (M\text{-}L^1)$$
$$(L^2) = C_L - (M\text{-}L^2), \text{ and}$$

$$K^*_{rel} = \frac{(M\text{-}L^2)\cdot[C_L - (M\text{-}L^1)]}{(M\text{-}L^1)\cdot[C_L - (M\text{-}L^2)]}$$

If we let a = the fraction of the copper which is associated with L^2 and f = the fraction of copper which is not bound to a ligand, then

$$(M\text{-}L^2) = a\,C_M$$
$$(M\text{-}L^1) = (1\text{-}f\text{-}a)\,C_M.$$

Since (1-a) >> f,

$$(M\text{-}L^1) = (1\text{-}a)\,C_M.$$

If we let $C_M = b \cdot C_L$, then

$$K^*_{rel} = \frac{a\cdot[1-(1-a)\cdot b]}{(1-a)\cdot(1-a\cdot b)}$$

where

a = fraction of copper bound to L^2
b = C_M/C_L.

For this case, a = 0.6909. When 0.1 equivalents of metal are present; i.e., when b = 0.1,

$$K^*_{rel} = 2.327.$$

Using McGovern's data for the Cpd II-copper(II) complex along with the formation constants for copper(II) and ammonia--the buffer used for these experiments--the conditional complex formation constant ($K^*_{Cpd\ II\text{-}Cu}$) was calculated to be $1.20 \times 10^{15}\ M^{-1}$, and

$$K^*_{\underline{27}\text{-}Cu} = K^*_{Cpd\ II\text{-}Cu} \times K^*_{rel} = 2.79 \times 10^{15}\ M^{-1}.$$

For 1a and copper, x=2 and n=1. Therefore, the relative conditional binding constant expands to

$$K^*_{rel} = \frac{(M\text{-}L^2)(L^1)}{(M\text{-}L^1)(L^2)(M)}$$

where

$(M\text{-}L^1)$ = the concentration of 27-copper complex
$(M\text{-}L^2)$ = the concentration of 1a-copper complex
(L^1) = the concentration of 27
(L^2) = the concentration of 1a
(M) = the concentration of uncomplexed copper(II).

Since $C_L1 = C_L2 = 33.3\ \mu M$, let $C_L1 = C_L2 = C_L$. Then the mass balance equations can be written as

$$C_M = (M) + (M\text{-}L^1) + 2\cdot(M_2\text{-}L^2)$$
$$C_L = (L^1) + (M\text{-}L^1)$$
$$C_L = (L^2) + (M_2\text{-}L^2).$$

Rearranging these equations into the forms

$$(M) = C_M - (M\text{-}L^1) - 2\cdot(M_2\text{-}L^2)$$
$$(L^1) = C_L - (M\text{-}L^1)$$
$$(L^2) = C_L - (M_2\text{-}L^2) \text{ and substituting,}$$

$$K^*_{rel} = \frac{(M_2\text{-}L^2)\cdot[C_L - (M\text{-}L^1)]}{(M\text{-}L^1)\cdot[C_L - (M_2\text{-}L^2)]\cdot(M)}.$$

If we let a = the fraction of the copper which is associated with L^2 and f = the fraction of copper which is not bound to a ligand, then

$$(M) = f\cdot C_M$$
$$(M\text{-}L^1) = a\cdot C_M$$
$$(M_2\text{-}L^2) = [(1\text{-}f\text{-}a)/2]\cdot C_M.$$

Since (1-a) >> f,

$$(M_2\text{-}L^2) = [(1\text{-}a)/2]\cdot C_M.$$

If we let $C_M = b\cdot C_L$, then

$$K^{*}_{rel} = \frac{(1-a)(1-ab)}{a[2-(1-a)b](M)}$$

where

a = fraction of copper bound to L^2
b = C_M/C_L.

For this case, a = 0.0823. When 0.1 equivalents of metal are present; i.e., when b = 0.1,

$$K^{*}_{rel} = 5.795/(M).$$

The concentration of free copper in solution can be calculated from the conditional complex formation constant for the 27-copper complex as follows

$$K^{*}_{27\text{-}Cu} = \frac{(27\text{-}Cu)}{(27)\cdot(Cu)} \Longrightarrow (Cu) = \frac{(27\text{-}Cu)}{(27)\cdot K^{*}_{27\text{-}Cu}}$$

Since

$$(27\text{-}Cu) = a\cdot C_M$$
$$(27) = C_{27} - a\cdot C_M = 10\cdot C_M - a\cdot C_M$$
$$K^{*}_{27\text{-}Cu} = 2.8 \times 10^{15}\ M^{-1},$$

$$(Cu) = \frac{a\cdot C_M}{[(10-a)C_M]\cdot K^{*}_{27\text{-}Cu}} = \frac{0.0823}{(10 - 0.0823)\cdot 2.79 \times 10^{15} M^{-1}}$$

$$= 2.96 \times 10^{-18}\ M.$$

Therefore,

$$K^{*}_{rel} = 1.96 \times 10^{18}\ M^{-1}, \text{ and}$$

$$K^{*}_{1a\text{-}Cu_2} = K^{*}_{27\text{-}Cu} \times K^{*}_{rel} = 5.47 \times 10^{33}\ M^{-2}.$$

Once arriving at K^{*} for the 1a-Cu_2 complex, it remains only to measure K^{*} for the 1a-Th_2 complex. In this a problem arises, again due to the fact that spectral changes upon metal binding are small. In addition, copper and thorium cause about the same degree of change. Therefore

we needed to distinguish between the two metal complexes or the two free metals. We were able to accomplish this using the complexometric reagent 1-[2-(5-chloropyridyl)azo]-2-naphthol (Cl-PAN, figure 23). Synthsized by the method of Shibata et al. (72), Cl-PAN is practically insoluble in water but very soluble in a variety of organic solvents, including ethanol, acetone, chloroform, dioxane, carbon tetrachloride, diethyl ether, and dimethylformamide (73, 74). Depending upon the conditions, Cl-PAN has been used to quantitatively detect a large number of metals. The free chromophore exhibits a yellow color, its λ_{max} occurring at 470 nm. Upon addition of thorium there is an almost imperceptible change to a yellow-orange color, indicating formation of the 4:1 Cl-PAN-thorium complex. This change is insufficient for quantitation of thorium. However, when copper is added to this reagent a wine red color is observed (λ_{max} = 550 nm), indicating formation of the 2:1 complex (75) of Cl-PAN with copper.

When copper was added to 70.9 μM Cl-PAN, the absorbance at 560 nm was linear when plotted versus the analytical concentration of copper, figure 30. When equimolar amounts of copper and thorium were added, the observed spectra are identical to those observed in the absence of thorium. Therefore, with Cl-PAN one can quantitatively measure free copper when free thorium is present in the same solution. The thorium is bound by Cl-PAN, but does not significantly alter the absorbance of the solution at 560 nm, as long as enough Cl-PAN is present to fill all the coordination sites of the metal present. If one assumes that all free copper is bound by Cl-PAN then the concentration of added copper is linearly related to the absorbance at 560 nm.

Since Cl-PAN is water insoluble and 1a is insoluble in benzene, an equilibrium was established in which 1a remains in the basic buffer while Cl-PAN remains in benzene. Any metal which is not bound by 1a will be extracted into the benzene layer by Cl-PAN. In this way 1a will not interfere with the absorbance of Cl-PAN, figure 31. To the biphasic system composed of pH 9.2 buffer and benzene was added Cl-PAN, copper, and varied amounts of 1a (0 to 0.5 equivalents relative to copper). If one plots the absorbance at 560 nm versus the analytical

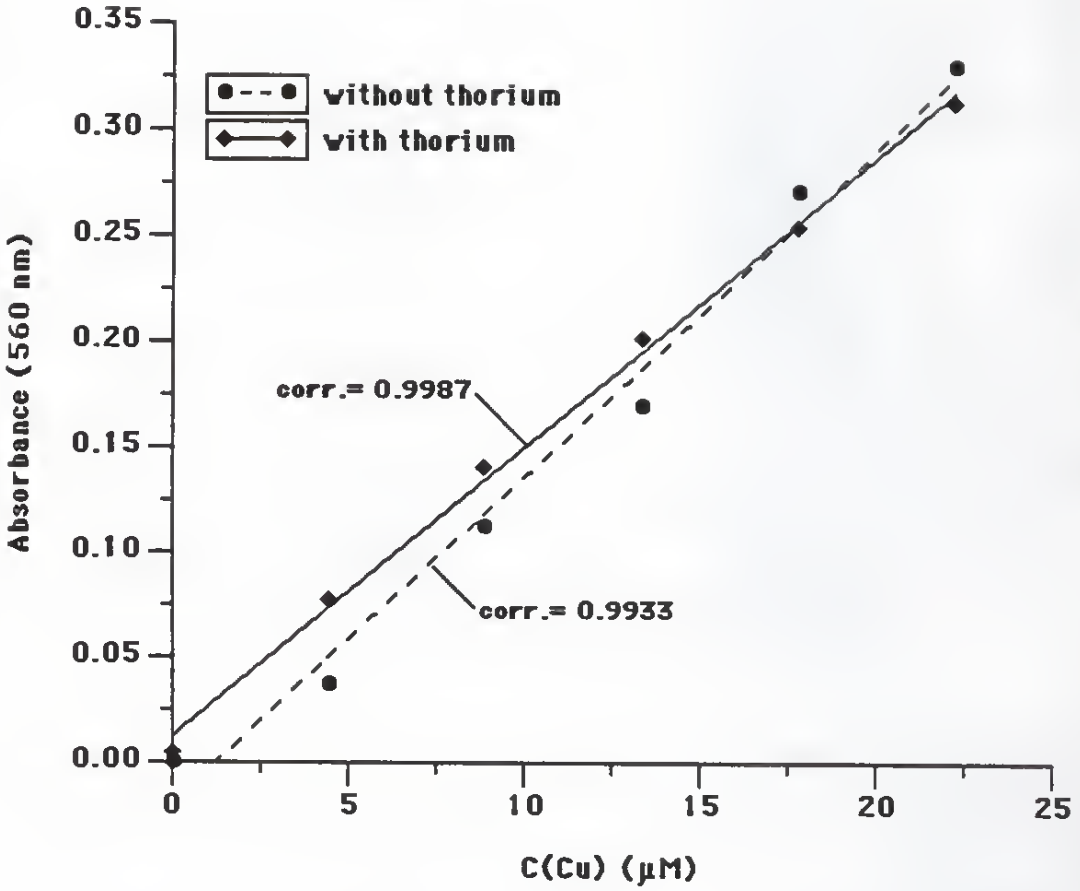


FIGURE 30
Standard Curve for Copper(II)Acetate with Cl-PAN (70.93 µM) in the Absence and Presence of Thorium(IV)Chloride at the Same Concentration as Copper.

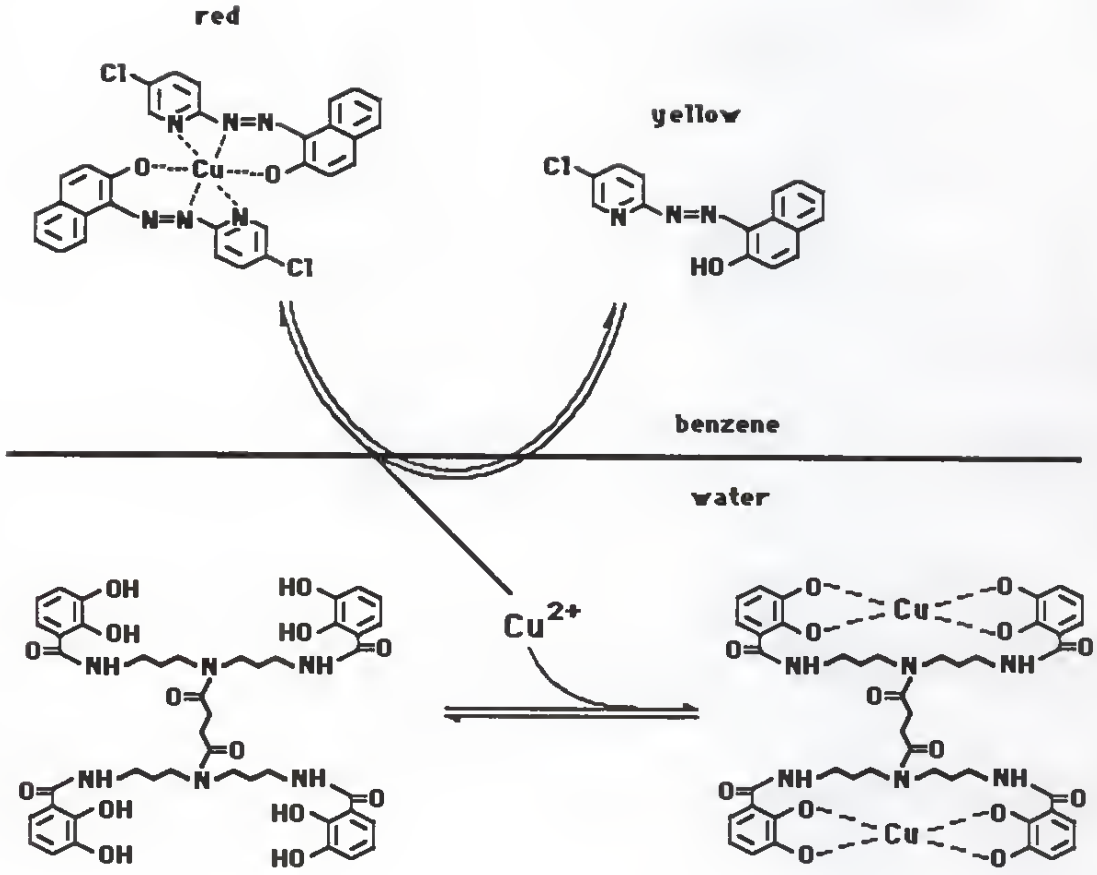


FIGURE 31
Equilibrium for Cl-PAN Copper Extraction.

concentration of 1a, a linear relationship exists, where more 1a causes less (Cl-PAN)$_2$-Cu to form, resulting in a decrease in the absorbance at 560 nm, figure 32. When an equivalent of thorium is present along with copper, the change of absorbance is decreased, indicating formation of the 1a-Th$_2$ complex, which results in more extracted copper and less decrease in the absorbance at 560 nm, figure 33. By treatment of the data in a fashion analogous to the two-ligand-one-metal competition experiment, x=2 and n=1, and the relative conditional binding constant expression expands to

$$K^{*}_{rel} = \frac{(Th_2\text{-}1a)\cdot(Cu)^2}{(Cu_2\text{-}1a)\cdot(Th)^2}$$

Since $C_{Th} = C_{Cu} = 33.3\ \mu M$, let $C_{Th} = C_{Cu} = C_M$. Then the mass balance equations can be written as

$$C_M = (Th) + 2\cdot(Th_2\text{-}1a)$$
$$C_M = (Cu) + 2\cdot(Cu_2\text{-}1a)$$

and rearranged into the forms

$$(Th) = C_M - 2\cdot(Th_2\text{-}1a)$$
$$(Cu) = C_M - 2\cdot(Cu_2\text{-}1a)$$

If we let a = the fraction of 1a associated with copper, then

$$(Cu_2\text{-}1a) = a\cdot C_{1a}$$
$$(Th_2\text{-}1a) = (1\text{-}a)\cdot C_{1a}\ , \text{and}$$
$$(Cu) = C_M - 2\cdot a\cdot C_{1a}$$
$$(Th) = C_M - 2\cdot(1\text{-}a)\cdot C_{1a}.$$

If we let $C_{1a} = b\cdot C_M$, then

$$(Cu_2\text{-}1a) = a\cdot b\cdot C_M$$
$$(Th_2\text{-}1a) = (1\text{-}a)\cdot b\cdot C_M$$
$$(Cu) = C_M - 2\cdot a\cdot b\cdot C_M$$
$$(Th) = C_M - 2\cdot(1\text{-}a)\cdot b\cdot C_M\ , \text{and}$$

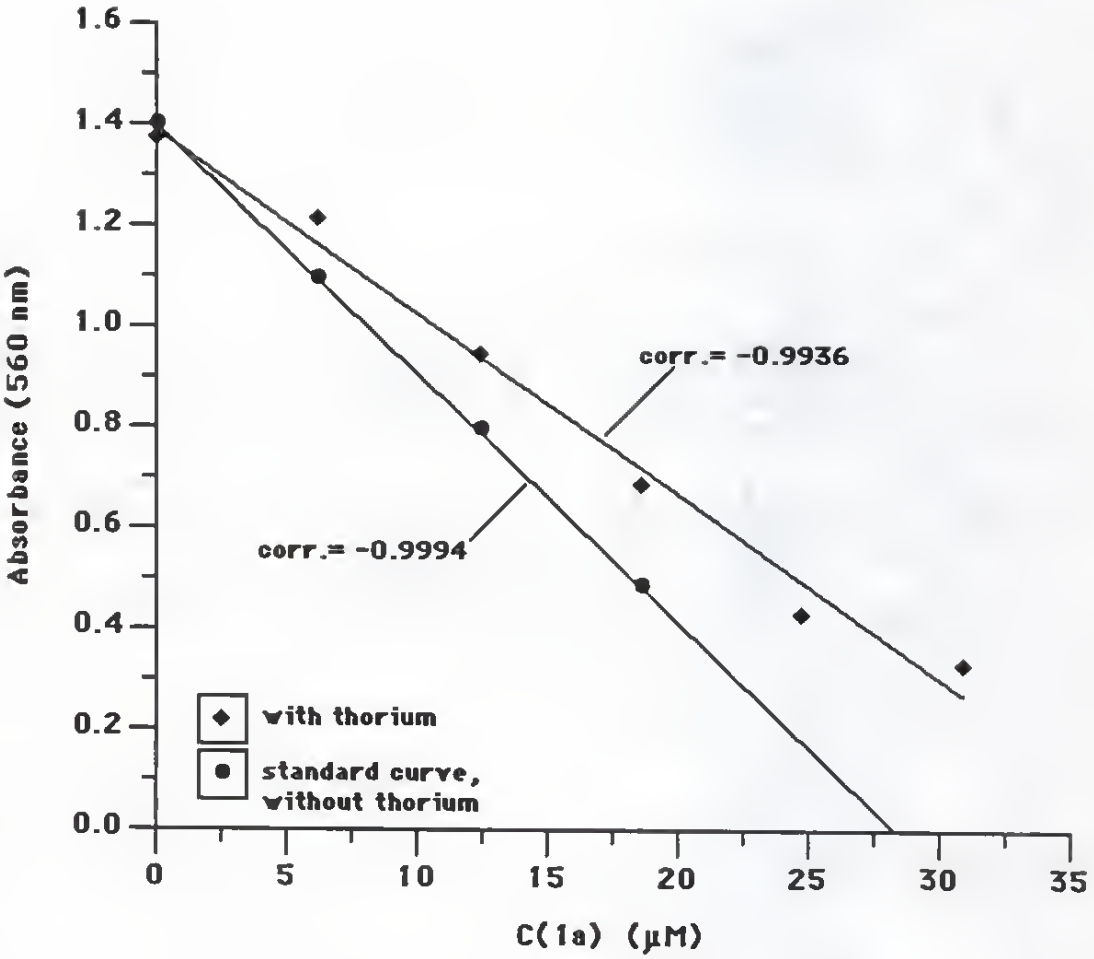


FIGURE 32
Results of 1a-Thorium-Copper Competition Experiments.

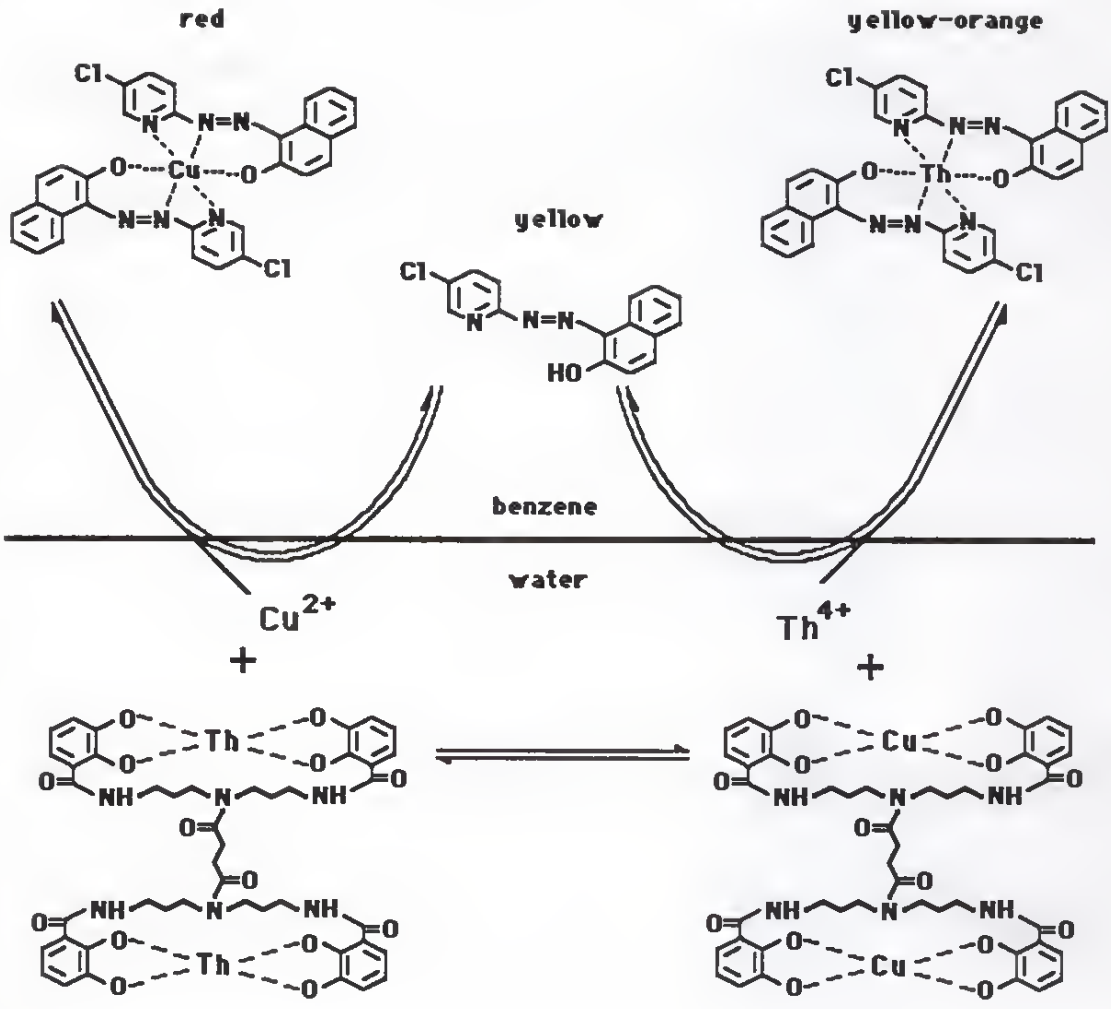


FIGURE 33
Equilibrim for Cl-PAN Copper/Thorium Extraction.

$$K^*_{rel} = \frac{(1-a)\cdot(1-2ab)^2}{a\cdot(1+2ab-2b)^2}$$

For this case, a = 0.7414. When 0.1 equivalents of 1a are present; i.e., when b = 0.1,

$$K^*_{rel} = 0.2814, \text{ and}$$

$$K^*_{1a\text{-}Th_2} = K^*_{1a\text{-}Cu_2} \times K^*_{rel} = 1.54 \times 10^{33}\ M^{-2}.$$

These results are summarized in table 9.

Pharmacology

Vehicle Development

It was observed that the catecholamide ligands are very soluble in alchohols and soluble at millimolar concentrations in ethyl acetate and nitrobenzene. Unfortunately, most organic solvents are not acceptible as vehicles for injection. As previously stated, ligand solubility in aqueous media at physiologic pH was slight. In order to solublize our ligands in physiologic buffer, Chremophor RH 40 (BASF) was used. This is a FDA-approved surfactant whose generic name is glycerol polyethylene glycol oxystearate. It is used commercially for solubilization of fat soluble vitamins, essential oils, and some hydrophobic pharmaceutical preparations. The LD_{50} for mice injected intraperitoneally is >6.4 g/kg.

Compounds were solubilized by maintaining a mixture of ligand and Chremophor at 55°C with frequent ultrasonic mixing. Once the mixtures were homogeneous in appearance--after one to two hours of this treatment--phosphate buffered saline at pH 7.4 (PBS) was added until the final solution was 40 percent Chremophor/PBS. These solutions were rather viscous, but suitable for injection.

TABLE 9
Results of Conditional Binding Constant Calculations for an H-Shaped Ligand and $ThCl_4$.

Ligand(s)	Metal(s)	Fraction Bound By First Competitor	K^*_{rel}[a]	Conditional Binding Constant for the First Competitor
Cpd II	Cu	—	—	$1.2 \times 10^{15}\ M^{-1}$
27/Cpd II	Cu	0.6909	2.327	$2.79 \times 10^{15}\ M^{-1}$
1a/27	Cu	0.9177	$1.96 \times 10^{18}\ M^{-1}$	$5.47 \times 10^{33}\ M^{-2}$
1a	Th/Cu	0.2586	0.2814	$1.54 \times 10^{33}\ M^{-2}$

[a]Calculated for one equivalent of each competitor and 0.1 equivalent of the substance for which they are competing, in 1.00 F ammonia buffer, pH 9.2.

Biological Toxicity

Any therapeutic device must be nontoxic, a situation not realized with other octacoordinate actinide chelators. In keeping with this concern all octacoordinate H-shaped ligands were tested for acute toxicity. The compounds were prepared by dissolving them in a mixture of Chremophor and PBS as previously described. A/J male mice (five mice per dose) weighing 20-25 grams were given intraperitoneal injections of these samples up to and including doses as high as 1000 µg/kg. It should be noted that this concentration is equivalent to administering 70 grams of drug to the average human, much more than the anticipated therapeutic dosage. The compounds were administered to five mice at each concentration. All mice seemed healthy and active and remained that way until the end of the 72 hour experiment, at which time the animals were sacrificed. Vehicle-injected mice exhibited no symptoms of toxicity.

Clearance Studies

Whether our H-shaped catecholamide chelators can remove actinides from animals with internal contamination is a question of paramount importance. To effectively perform this role the compounds must be able to selectively sequester actinides from body tissues, proteins, and fluids, which may themselves bind the metals. It is no mean task for the chelator to selectively bind an actinide metal in a millieu replete with various cations and endogenous sequestering agents. Next, the chelate must be able to utilize an excretion route. This was a problem in the case of Raymond's CYCAM series, where the chelators bound plutonium but were not excreted (44).

The following experiments represent preliminary attempts to ascertain the ability of catecholamide chelators to promote actinide excretion. No attempt has been made to optimize dosing. The intention was to test the concept of using H-shaped octacoordinate catecholamide chelators to remove actinides in the same way that hexacoordinate catecholamide chelators have been shown to eliminate iron.

Urinary clearance

The ability of DTPA, Compound II, and H-shaped ligands to stimulate clearance of thorium in the urine was evaluated. Thorium (1 nmole in 0.5 mL in 0.09 percent citrate buffer) was first administered to rats by intraperitoneal injection. One hour later a chelating agent (200 μmoles/kg) was administered intraperitoneally in the 40 percent Cremophor/PBS vehicle. Animals were placed in metabolic cages with food and water *ad libitum*. Urine samples were collected at six hour intervals and assayed for thorium by scintillation counting of the metal's alpha radiation. The data indicate that DTPA is effective at stimulating thorium excretion in the urine, and that this process is very rapid, figure 34. The fact that most of the DTPA-stimulated clearance takes place within the first six hours is consistent with the short half-life (20 minutes) of this drug in the body. Although the catecholamide chelators tested in this way (1c-f and 1m) stimulated thorium output in the urine to a very small extent, if we make the assumption that additional methylenes increase lipophilicity, a structure-activity relationship exists between the lipophilicity of the ligand and the percent urinary thorium excretion. As one would expect, the analog presumed to be the least lipophilic, 1d, stimulated the most urinary excretion while 1e was the least effective.

To determine if a connection exists between this result and the lipophilicity or degree of ionization of the ligand, the partition coefficient for two ligands--1e and 1f--were measured in octanol/phosphate buffered saline, pH 7.4 (PBS). As expected, 1e was found to be more lipophilic than 1f, its partition coefficient being 1.63 times greater, table 10.

Fecal clearance

The inability of our catecholamide actinide chelators to stimulate thorium excretion via the urine came as no great surprise. It has been shown that highly lipophilic chelators are excreted as their plutonium complexes almost exclusively in the feces (76). Our ligands, of course, fall into this lipophilic class. Furthermore, experience with hexacoordinate catecholamide iron chelators has shown that these lipophilic compounds are also excreted mainly in the feces (77).

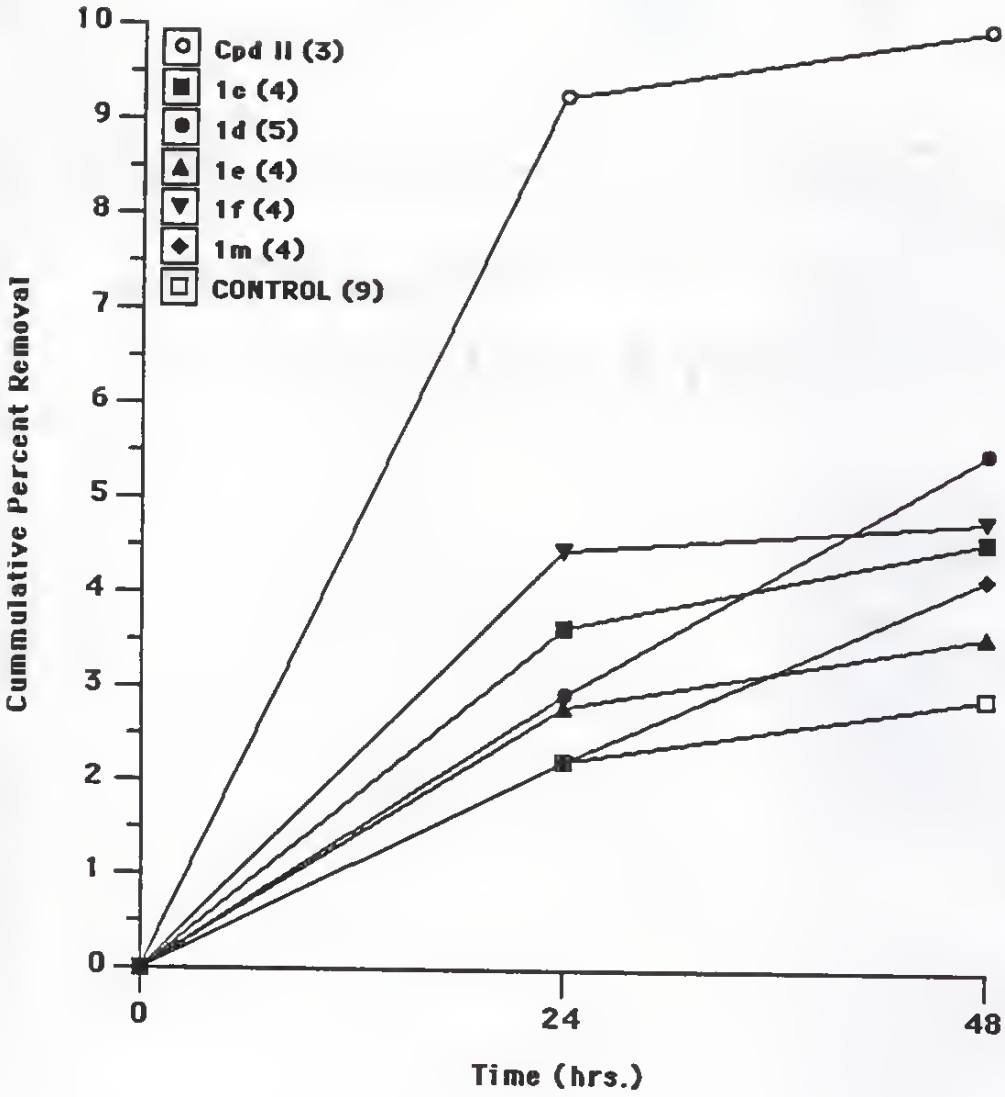


FIGURE 34

Effects of Catecholamide Chelators on the Urinary Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 200 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

TABLE 10
Results of Partition Coefficient Measurements for Ligands 1e and 1f.

Compound	λ_{max}		$\varepsilon_{320\,nm}$		$\frac{[ligand]_{octanol}}{[ligand]_{PBS}}$
	octanol	PBS	octanol	PBS	
1e	313 nm	317 nm	12,800	13,320	39.96
1f	313 nm	319 nm	12,840	13,040	24.50

Considering the structural similarities of our catecholamide actinide chelators to these iron chelators, and their greater molecular weight, one would predict that these compounds would be excreted by the fecal route as well.

The potential health hazard of ^{230}thorium, the isotope used for these studies, is well recognized; precautions and procedures for its safe use have been implemented to avoid contamination of individuals and working areas. Because of the necessary restrictions in handling thorium-containing samples, a safe technique for measuring the thorium content of feces has not been developed. Additionally, it is difficult to gather kinetic data from fecal samples, due to irregularity of sample excretion.

Biliary clearance

To avoid the handling difficulties associated with measuring actinide output in feces, we have developed a series of experiments to collect and assay biliary tisues and fluids before and after chelation therapy.

Gall bladder excision. As an initial experiment, 36 mice were administered thorium (50,000 dpm) intraperitoneally, followed one hour later by an H-shaped ligand (200 µmoles/kg in 0.5 mL 40 percent Chremophor/PBS). At three hour intervals, 3 mice were sacrificed, and their gall bladders were removed and assayed for thorium content. Since the gall bladder acts only as a bile storage site, the amount of thorium in the gall bladder would depend on the diet composition, the amount consumed, and when it was consumed. This experiment will therefore only qualitatively indicate whether or not biliary excretion of thorium can be stimulated by H-shaped ligands. Mice that recieved an H-shaped ligand demonstrated approximately a three-fold increase in the thorium content of their gall bladders, figure 35. This data suggests that our chelators are excreted by the biliary route, and are effective actinide chelators.

Bile duct cannulation. In order to get quantitative excretion data and accurate kinetic data it was decided to collect bile for several days. This was accomplished by placing an indwelling cannula into the bile duct of adult male Sprague-Dawley rats, allowing for continuous collection

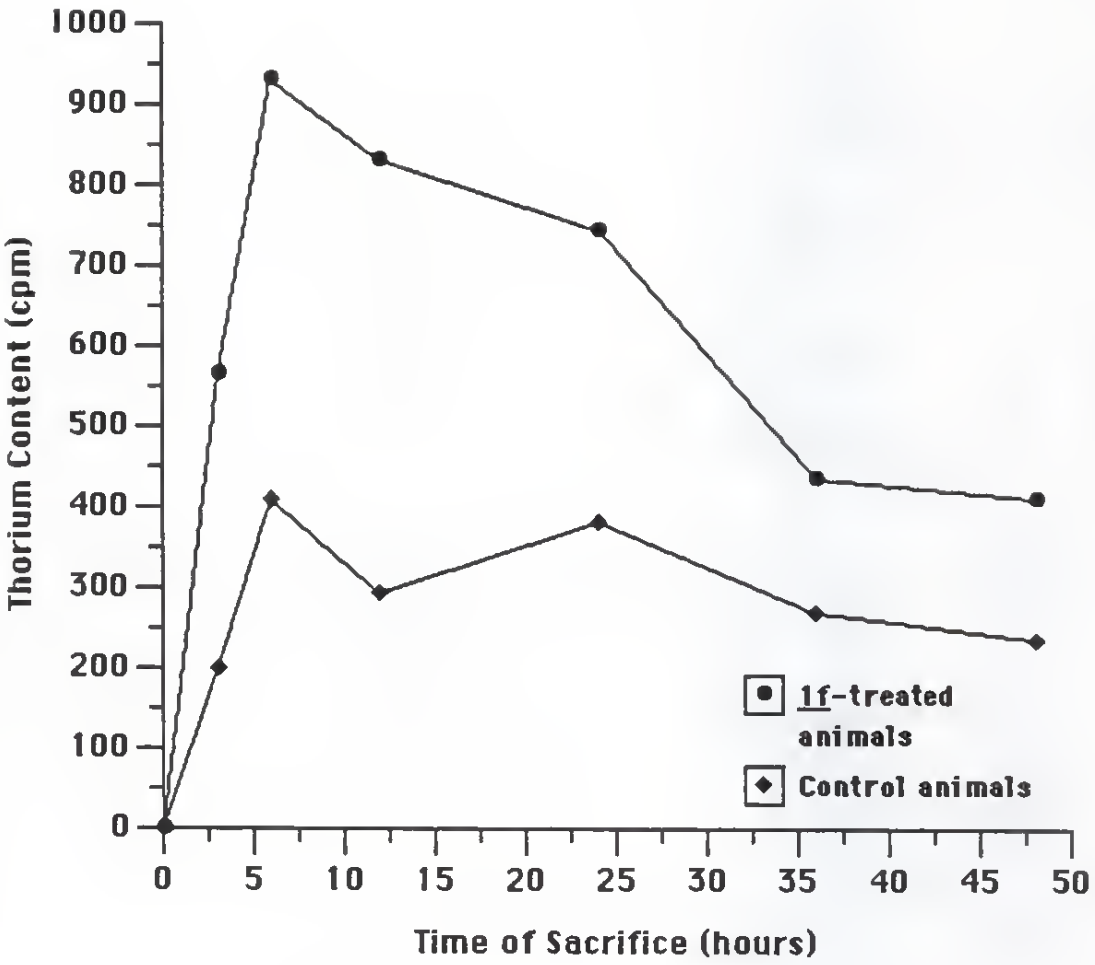


FIGURE 35
Effect of 1f on the Thorium Content of Mouse Gall Bladders Over 48 Hours, at a Dosage of 200 µmoles/kg. Chelators Were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium (50,000 cpm).

of bile. By using the rat, one does not face the problems associated with the gall bladder since this organ is absent. The cannula emerges from the incision and, using a skin-tunneling needle, is directed under the skin to behind the rat's neck. The emerging cannula is directed through a torque-transmitting spring tether to a fluid swivel located above the metabolic cage. The animal is able to move about freely in the cage while urine and bile were collected. Urine was collected for 24 hour intervals while bile was collected for three hour intervals. Although more involved, this technique allows us to obtain very reproducible data for metal excretion. When the animal began to recover from anethesia and good bile flow had been established, thorium (1 nmole) was administered intraperitoneally. One hour later a chelator was given also intraperitoneally (100 or 200 μmoles/kg). Surgical techniques were performed by Dr. N. L. Scarborough, Dr. S. A. Prudencio, and Ms. Kady Crist. I assisted in sample preparation for scintillation counting.

To study the effect of altering the spermidine portion of H-shaped ligands, 1d, 1e, and 1f were tested. It was found that the assymmetric SPD backbone is much more effective at stimulating thorium elimination via the bile than either symmetric backbone, figure 36. Since both addition to and removal from the spermidine backbone of a single methylene group causes a significant decrease in biliary thorium removal, it is reasonable to assume that any further alterations in a, b, c, and d (figure 5) will result in a further decrease in thorium excretion.

To examine the role of the connecting diacid, 1c, 1f, and 1m were tested. The glutaryl moiety appears to be better than either the succinyl or adipoyl diacids. Therefore, for the same reasons, no other diacids were tested. Total thorium removal is shown in figure 37.

Combination chelator therapy

The data show that H-shaped ligands stimulate thorium excretion almost exclusively in the bile whereas DTPA leads to urinary clearance of the actinide. Based on these findings we felt that perhaps if both drugs were administered in combination, the total actinide clearance would increase. To test this concept, the bile duct cannulation procedure was employed. In this experiment, rats were administered thorium intraperitoneally (1 nmole) followed by DTPA (100

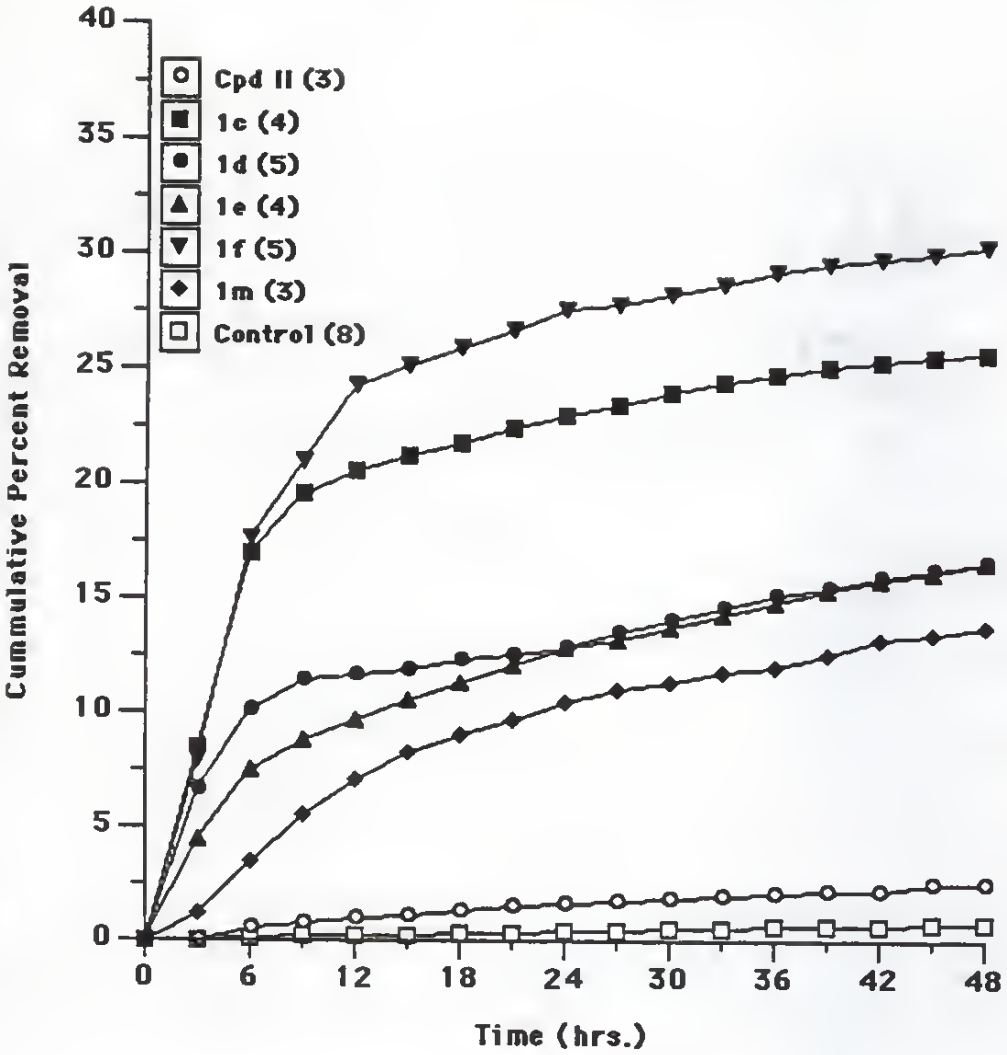


FIGURE 36
Effects of Catecholamide Chelators on the Biliary Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 200 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

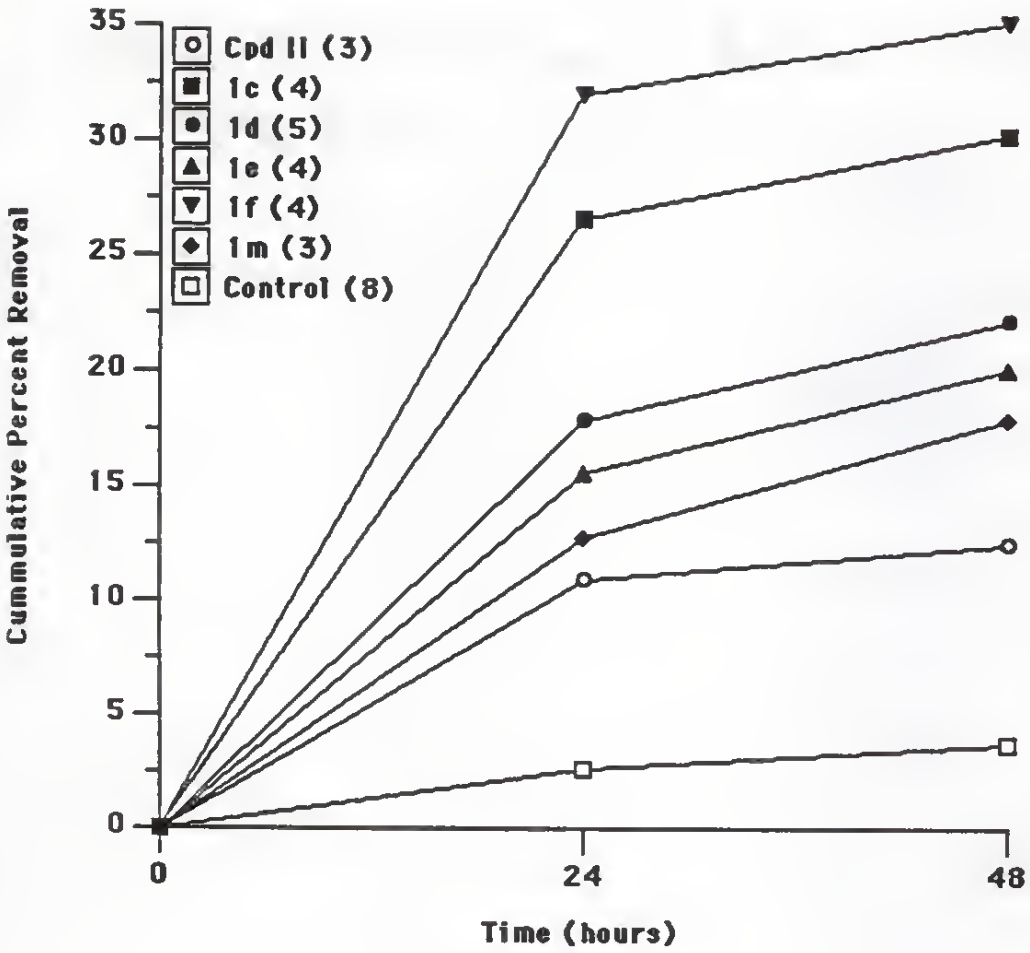


FIGURE 37
Effects of Catecholamide Chelators on the Total Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 200 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

µmoles per kg) and/or 1f (100 µmoles/kg), also intraperitoneally. Urine and bile were collected and assayed for thorium content, as previously described.

Urinary clearance. In the urine DTPA was able to remove 28.0 percent of the injected dose of thorium when administered alone at this lower dose, figure 38. When given in combination with 1f, only 22.3 percent of the injected dose was found in the urine, a 20.4 percent reduction.

Biliary clearance. In the bile a similar situation was observed, figure 39. 1f alone removed 23.6 percent of the injected dose of metal, but only 17.8 percent was removed in the bile by the combination of chelators, a 24.6 percent reduction.

Total clearance. The data may indicate that 1f does indeed access different body compartments than DTPA, as evidenced by increased thorium excretion. Also, it appears that there are some compartments that are frequented by both drugs, as reflected by the fact that the combination of drugs removes less than an additive amount of metal. If DTPA and 1f were independent, then the metal removed by DTPA in the urine when administered by itself added to the metal removed by 1f in the bile when administered by itself would be approximately equal to the total output when the drugs are administered in combination. Since the actual output of metal is 21.6 percent less than the additive amount, there is some overlap in the volumes of distribution for the two chelators. That is, there are some body compartments which are accessible to both chelators, some which are accessible to only one chelator, and some which are not accessible to either chelator. When administered in combination, in the compartments accessible to both chelators a portion of the available thorium is bound by each chelator. for example, some of the thorium which would have been bound by DTPA and eliminated in the urine is bound by 1f, causing an apparent decrease in the effectiveness of DTPA. Although combination therapy decreased the appparent effectiveness of each chelator, the total excretion did increase, figure 40.

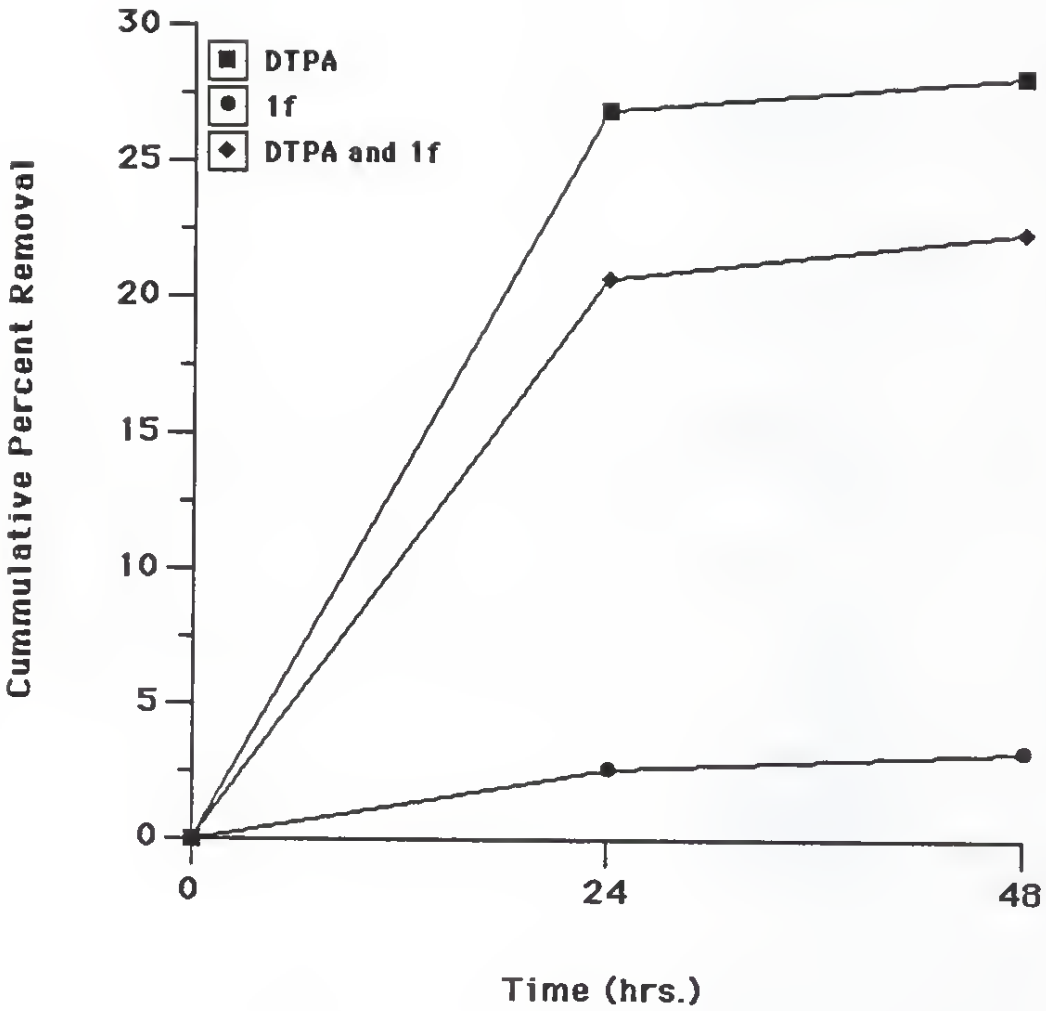


FIGURE 38
Effects of 1f or DTPA on the Urinary Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 100 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

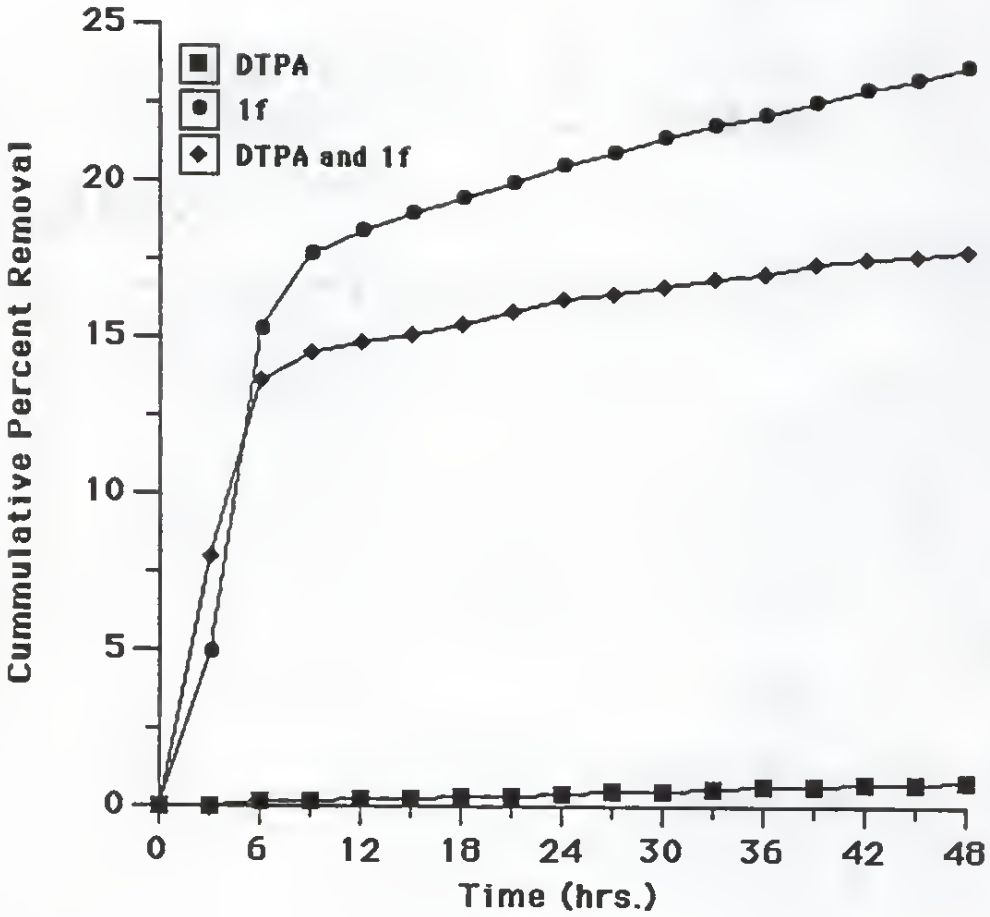


FIGURE 39
Effects of 1f or DTPA on the Biliary Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 100 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

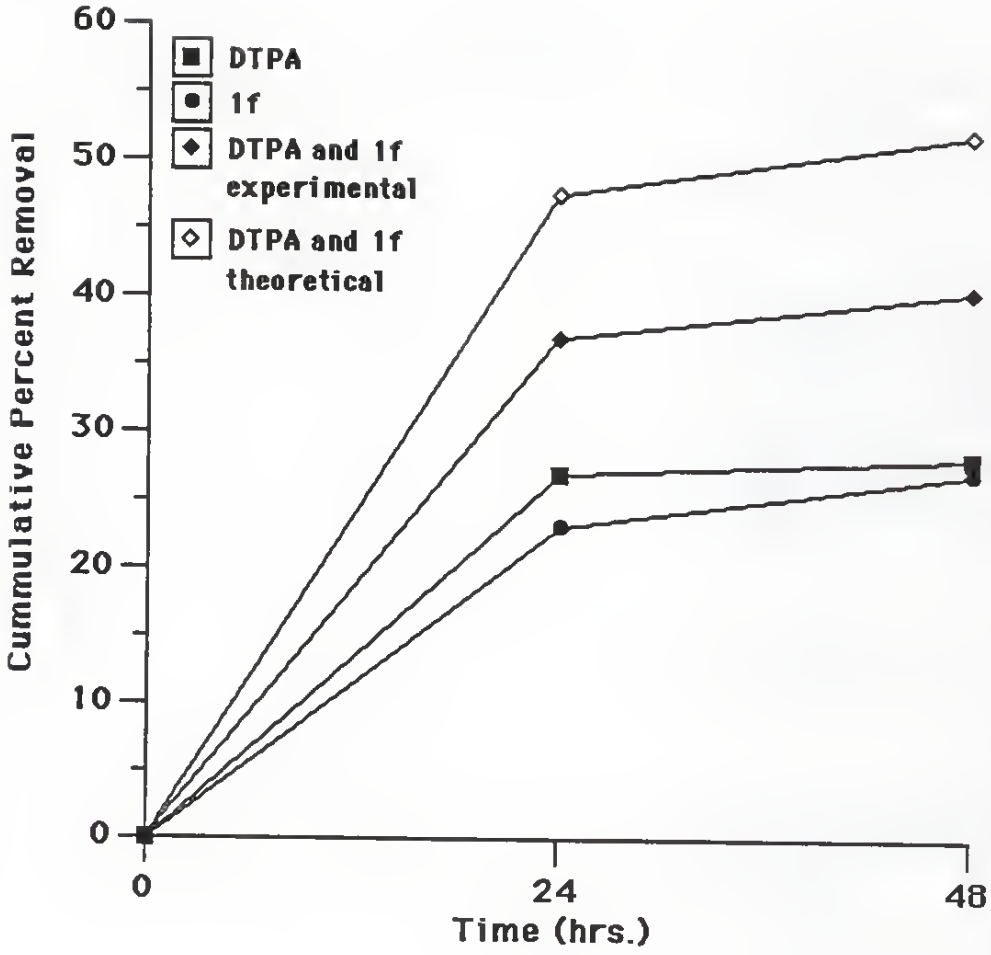


FIGURE 40

Effects of 1f or DTPA on the Total Excretion of Thorium in Rats Over 48 Hours, at a Dosage of 100 µmoles/kg each. Chelators were Administered Intraperitoneally One Hour After Intraperitoneal Injection of ^{230}Thorium. Numbers in Parentheses Represent the Number of Rats Tested.

CHAPTER III

ELUCIDATION OF THE SOLUTION STRUCTURE OF POLYAMINES IN RELATION TO THE MECHANISM OF CELLULAR UPTAKE

Background

Bergeron, Porter and Stolowich have provided a great deal of information regarding the structural requirements, preferences, and limitations associated with substrates of the polyamine transport apparatus (7, 8, 9, 10, 78, 79).

Backbone Variations

Eleven structural analogs of spermidine and putrescine were synthesized and studied both for their ability to inhibit [^{3}H]-spermidine uptake by the polyamine transport apparatus of L1210 cells and/or their ability to stimulate growth *in vitro* in polyamine-depleted L1210 cancer cells, table 11. One should note that the analogs used in this study differ from the naturally-occurring polyamines only in the number of methylene units separating the various amine functionalities. Uptake inhibition was found to be competitive in all cases, homospermidine being the most competitive polyamine. Any further alterations in the number of methylene units in the polyamine backbone resulted in diminished competitive inhibition. Replacement of the secondary nitrogen of spermidine by a methylene unit produced a molecule which was an effective inhibitor of [^{3}H]-spermidine uptake. In growth stimulation experiments, however, little effect was caused by these analogs. This study suggests that the secondary amine is essential for polyamine functions while its importance is less pronounced for uptake.

N^4-Substitution

Alkylation. N^4-alkylated spermidine derivatives were synthesized to determine both how N^4-alkylation would affect uptake characteristics and how large of an alkyl substituent would be tolerated by the polyamine transport apparatus, table 12. It was found that the spermidine

TABLE 11
Inhibition of [^{3}H]-Spermidine Uptake into L1210 Cells by Polyamines or Their Homologs.

Homolog	K_i[a] (μM)	[^{3}H]-Spermidine Uptake Inhibition[b] Picomoles per 10^7 cells-min	Percent of Control
none	—	56.1	100
DA_3[c]	>500	54.0	96
DA_4(putrescine)	171.3	44.6	80
DA_5	459.0	54.0	96
DA_6	63.2	40.1	71
DA_7	18.2	23.0	41
DA_8	22.1	25.2	45
$_3TA_3$[d]	8.4	16.1	29
$_4TA_4$	3.5	7.3	13
$_3TA_5$	12.3	19.8	35
$_3TA_6$	13.1	19.6	35
$_3TA_7$	13.0	20.0	36
$_3TA_8$	7.8	13.5	24
Spermine	9.1	17.1	30

Reference 79.

[a]Prewarmed L1210 cells($5x10^6$) were incubated for 20 min in 1 mL of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 μM [^{3}H]-spermidine and 100 μM homolog. Uptake data were fitted by computer for competitive inhibition; the Michaelis constant for spermidine uptake was 2.0 μM, and the maximum velocity of the reaction was 117 pmole/min per milligram of protein.

[b]Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]-spermidine plus100 μM putrescine or spermidine homolog.

[c]The abbreviation for putrescine homologs having the general structure $NH_2(CH_2)_nNH_2$ is DA_n (for diamine) where n=3 to 8.

[d]The abbreviation for spermidine homologs having the general structure $NH_2(CH_2)_nNH(CH_2)_{n'}NH_2$ is $_nTA_{n'}$ (for triamine) where n is 3 or 4 and n' is 3 to 8.

TABLE 12
Effects of N^4-Alkylated Spermidines on [^{3}H]-Spermidine Transport into Ascites L1210 Leukemia Cells.

Substituent	Relative Uptake[a]	K_i (μM)[b]
-H	100	---
$-CH_3$	17	3.4
$-CH_2-CH_3$	14	3.1
$-(CH_2)_5-CH_3$	69	34
$-CH_2-C_6H_5$	67	39

Reference 79.

[a]Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]-spermidine plus100 μM analog.

[b]Prewarmed L1210 cells($5x10^6$) were incubated for 20 min in 1 mL of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 μM [^{3}H]-spermidine and 10 or 100 μM analog.

molecule could be alkylated rather extensively at the N^4-position and still participate effectively in experiments related to uptake, including inhibition of [^{3}H]-spermidine uptake, prevention of MGBG-induced cytotoxicity, and intracellular detection by HPLC. MGBG, methylglyoxal-bis-guanylhydrazone, is an inhibitor of polyamine biosynthesis.

Acylation. N^4-acylation had more pronounced effects upon uptake than did alkylation. A series of N^4-acyl spermidine derivatives and their alkyl counterparts--for example, ethyl and acetyl--were prepared and evaluated for their ability to compete with [^{3}H]-spermidine for cellular uptake, table 13. It was found that both series of derivatives could behave as polyamine transport apparatus substrates, but there was an obvious preference for the N^4-alkylated derivatives. In addition, the secondary nitrgen could be extensively modified without a significant decrease in inhibitory effect, as evidenced by the uptake of derivatives such as N^4-hexyl and N^4-benzyl spermidine. This is consistent with the conclusions of the previous study, where the primary amines appeared to be more critical for uptake.

N^1,N^8-Bis Substitution

A series of N^1,N^8-bis acyl derivatives were prepared and tested in a similar manner, table 14. Of the derivatives tested--N^1,N^8-bis-methyl and N^1,N^8-bis-formyl spermidine were not tested--N^1,N^8-bis ethyl spermidine was the only molecule capable of competing effectively with spermidine for uptake; any modification to this derivative rendered the molecule incapable of behaving as a polyamine transport apparatus substrate.

The Role of Protonation State: Potentiometric Measurements

In an attempt to more accurately define the role of charge in polyamine uptake, the protonation state of N^4-benzylspermidine, as well as the nor- and homospermidine analogs, was studied. To achieve this the pK_a of each nitrogen of the three analogs, and homospermidine itself was determined potentiometrically. The benzyl analogs were chosen because considerable uptake data has been accumulated on these compounds.

TABLE 13
Comparison Between the Effects of N^4-Alkylated and N^4-Acylated Spermidines on [^{3}H]-Spermidine Transport into Ascites L1210 Leukemia Cells.

Substituent	Relative Uptake[a]	K_i (μM)[b]
-H	100	—
$-CH_2-CH_3$	14	3.1
$-(C=O)-CH_3$	81	115
$-CH_2-(CH_2)_4-CH_3$	69	34
$-(C=O)-(CH_2)_4-CH_3$	88	135
$-CH_2-C_6H_5$	67	39
$-(C=O)-C_6H_5$	92	500

Reference 79.

[a]Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]-spermidine plus100 μM analog.

[b]Prewarmed L1210 cells($5x10^6$) were incubated for 20 min in 1 mL of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 μM [^{3}H]-spermidine and 10 or100 μM analog.

TABLE 14
Effects of Terminally Modified Spermidines on [^{3}H]-Spermidine Uptake into L1210 Leukemia Cells.

Substituent	Relative Uptake[a]	K_i (μM)[b]
-H	100	---
-(C=O)-CH_3	91	508
-CH_2-CH_3	69	62
-(C=O)-CH_2CH_3	92	550
-CH_2-CH_2CH_3	80	117
-tBOC	91	521
-2,3-Dimethoxybenzoyl	91	256

Reference 79.

[a]Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]-spermidine plus100 μM analog.

[b]Prewarmed L1210 cells($5x10^6$) were incubated for 20 min in 1 mL of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 μM [^{3}H]-spermidine and 10 or100 μM analog.

When an aqueous titration was completed, a set of data consisting of pH measurements versus volume of titrant added was generated. The computer program PHFIT (D. Leussing, private communication) was used to analyze this data. PHFIT handles as many as four independent species and fifteen associated species. The program first calculates, by way of a standard Newton-Raphson iteration, the distribution of species at each data point based on the total concentration of species at that point and the estimated pK_a provided for each specie. Thus, an initial theoretical titration curve is obtained; assuming all significant equilibria have been considered, the difference between this calculated curve and the observed data is then minimized by further refinement of the initial estimates. To calculate the acid dissociation constants, one must provide estimates for these constants, the analytical concentrations of all independent species involved in the chemical equilibria, the autodissociation constant (K_w) for water under the experimental conditions, and the activity coefficient (γ) of H^+ under the experimental conditions. Also, certain assumptions are made--that the titrations performed under conditions of constant temperature and ionic strength.

By using a water-jacketed cell in conjunction with a constant temperature water bath, the condition of constant temperature (25°C) is satisfied. To insure the condition of constant ionic strength, an excess of a strong electrolyte, potassium chloride, is added to both the solution of interest and the titrant, so that changes in the ionic state of the acid and titrant are insignificant in comparison to the total ion population. In practice this means that the ligand concentration (approximately 2×10^{-3} M) is much less than the ionic strength of the solution (0.1M KCl). For an aqueous solution at 25°C with an ionic strength of 0.1M, K_w and γ are $10^{-13.787}$ and 0.78 respectively (80, 81). These two terms are related by the expression

$$K_w=[H^+][OH^-]= \frac{a_{OH}\, a_H}{\gamma^2}$$

where a_H and a_{OH} are the activity of H^+ and OH^- respectively. The results of the pK_a studies are presented in table 15 along with other selected polyamine pK_a's.

Knowing the pK_a's, it is possible to calculate the concentration of the mono-, di-, and trication of a polyamine in solution at any given pH, using the equations

$$\alpha(0) = \frac{K_1K_2K_3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha(1) = \frac{K_1K_2[H^+]}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha(2) = \frac{K_1[H^+]^2}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3}$$

$$\alpha(3) = \frac{[H^+]^3}{[H^+]^3 + K_1[H^+]^2 + K_1K_2[H^+] + K_1K_2K_3},$$

where $\alpha(i)$ is the fraction of polyamine existing as the i+ cation. Recall that the order of uptake inhibition is homospermidine > spermidine > norspermidine. It is clear that there are substantial differences in the relative concentrations of polycations at a particular pH. For example, at pH 7.4 only 67.4 percent of norspermidine is in the form of the trication while 89.9 percent of spermidine is in this form and 97.1 percent of homospermidine is triprotonated, figure 41. Similar trends are seen with the benzyl compounds, where11.4 percent, 34.3 percent, and 69.1 percent, respectively, exist as the trication.

The values for $\alpha(3)$ seem to correlate well with C. W. Porter's *in vitro* measurements of inhibition constants (7, 8). By multiplying the actual concentration of polyamine by the fraction of the polyamine which exists as the trication at pH 7.4 one can determine the "effective" concentration of polyamine. When one recalculates the K_i's of various polyamines based on these corrected concentrations, the large differences in affinity for the transport apparatus are greatly diminished, table 16. However, the role of a 3+ cation is unclear in view of the fact that

TABLE 15

Results of Acid-base Titrations of NH_2-$(CH_2)_a$-N(C=X)-$(CH_2)_b$-NH_2

a	b	x	pK_1	pK_2	pK_3	# of Titrations[a]
3	3	H_2	10.579 (56)[b]	9.793 (78)	6.507 (66)	5
3	4	H_2	10.647 (29)	9.836 (66)	7.118 (100)	6
4	4	H_2	10.835 (32)	10.111 (63)	7.752 (135)	6
3	3	=O	9.979 (190)	7.763 (350)	---	5
3	4	=O	10.511 (64)	8.970 (76)	---	6

[a]Performed at 25°C in 0.1 M KCl, [polyamine] = 0.001 M.

[b]Numbers in parentheses represent the standard deviation associated with the last digit.

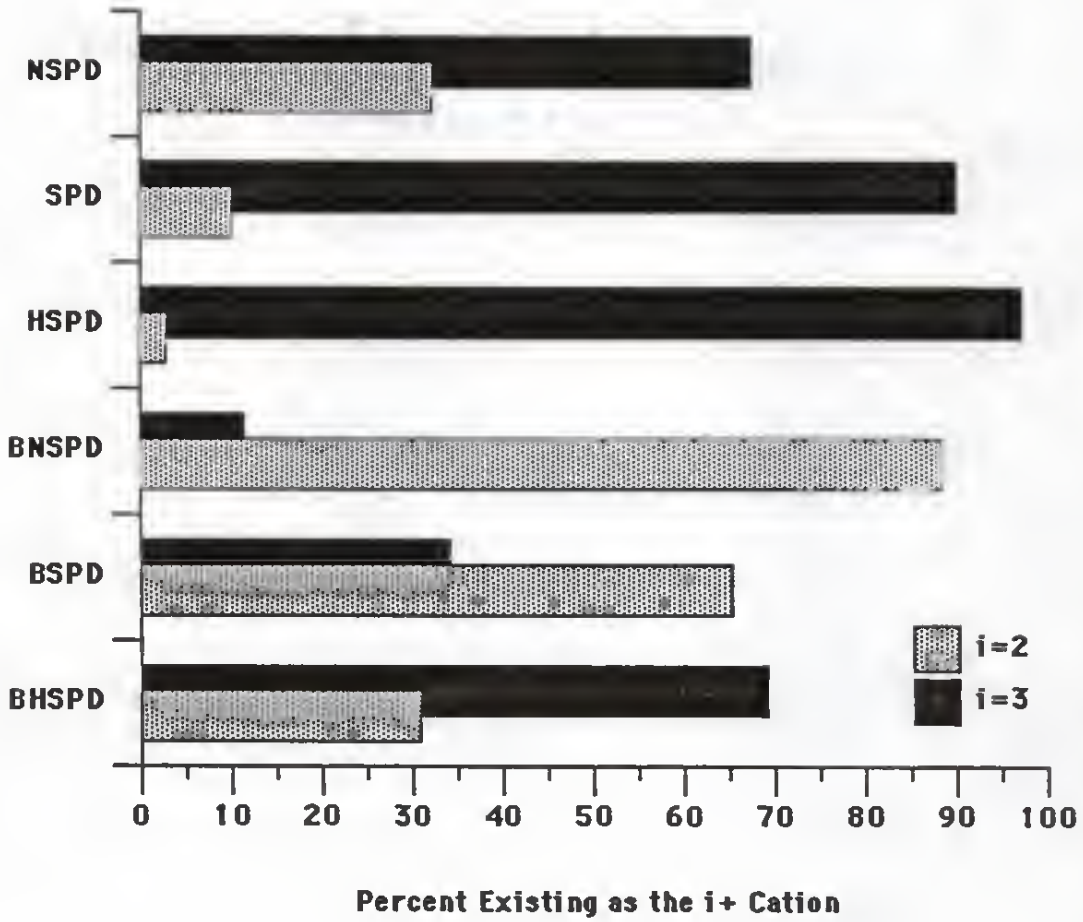


FIGURE 41
Distribution of Protonated Species at pH 7.4 for Several Triamines.

TABLE 16
Correlation Between the Polyamine Inhibition Constant for Spermidine Uptake and the Fraction of Polyamine Existing as the Trication at Physiological pH.

Compound	K_i[a]	$\alpha(3)$[b]	$K_i \times \alpha(3)$
BNSPD[c]	135 mM	0.114	15.4 mM
BSPD	36	0.343	12.3
BHSPD	14	0.691	9.7
NSPD	8.4	0.674	5.7
HSPD	3.5	0.971	3.4

[a]Reference 8.
[b]Calculated from measured pK_a's, see text.
[c]Abbreviations used are: BNSPD N^4-benzyl-norspermidine; BSPD N^4-benzyl-spermidine; BHSPD N^5-benzyl-homospermidine; NSPD norspermidine; HSPD homospermidine.

long chain diamines, which would be 2+ cations, compete so effectively for uptake. Furthermore, in looking at the concentration of the 2+ cation of triamines and competition with spermidine for uptake an inverse relationship seems to exist. Finally, there is so little 1+ cation and free base that their roles would seem insignificant. It may be that the role of the trication is to bring the polyamine to the surface of the negatively charged cell membrane. Once associated with the membrane, other factors may come into play. Clearly, charge is not the sole explanation for the selectivity in polyamine uptake.

The Role of Hydrogen Bonding

The Reasoning

Both the N^4-benzoyl and the N^4-acetyl compound have restricted rotation about the amide C-N bond while the corresponding reduced compounds have more freedom of rotation. This implies that although the position in which the acetyl group is held does not substantially hinder uptake, the benzoyl group does. A consideration of how charges on the terminal nitrogens of spermidine might be utilized to hold the polyamine in a particular conformation and the possible conformations which might allow a benzoyl versus a benzyl functionality--or, to a lesser extent, an acetyl versus an ethyl functionality--to interfere with this binding led us to postulate the importance of a hydrogen-bonded cyclic conformation for polyamine transport. It is clear that a trication cannot form intramolecular hydrogen bonds and that spermidine exists mainly as a trication in an aqueous environment at pH 7.4. It may be that the trication is energetically unfavorable at the cell membrane and that less charged species exist.

It has been shown that the strength of a hydrogen bond is maximal when the bond is colinear; i.e., when the X--H--X angle formed by the nuclei is 180 degrees. In the case of intramolecular hydrogen bonds the optimal angle cannot always be achieved, resulting in a weaker bond. For example, hydrogen bonding of the terminal hydroxyls of compounds of the general structure $HO-(CH_2)_n-OH$ have been measured by infrared spectroscopic analysis (82). The data indicates that 1,4-butanediol (n=4) forms a stronger intramolecular hydrogen bond

than does 1,3-propanediol (n=3), table 17. For terminal diamines H_2N-$(CH_2)_n$-NH_2 acid-base titrimetry has provided similar data; i.e., 1,4-diaminobutane is more basic than 1,3-diaminopropane (83). This may be due to increased electrostatic repulsion in the case of 1,3-diaminopropane and/or due to the presence of a stronger intramolecular hydrogen bond in the case of 1,4-diaminobutane; a seven-membered intramolecularly hydrogen bonded ring can more closely achieve a conformation which optimizes the N--H--N bond angle.

In previous uptake experiments, homospermidine inhibited [^{3}H]-spermidine uptake better than norspermidine, implying that the aminobutyl sidechain is more easily recognized by the polyamine transport apparatus than the aminopropyl sidechain. Since the aminobutyl sidechain can form a more stable intramolecular hydrogen bond, a greater percentage of homospermidine may exist as the cyclic hydrogen-bonded conformer on the cell surface, leading to better recognition and inhibition. Although in the case of N^1,N^7-diaminoheptane and N^1,N^8-diaminooctane no central nitrogen with which to form an intramolecular hydrogen bond exists, a psuedo-cyclic conformation may still be achieved by folding of these molecules. In addition, N^4-benzoyl spermidine cannot compete effectively for uptake, This may reflect the inability of this molecule to achieve a cyclic conformation for steric reasons. However, the corresponding benzyl compound can compete very well. Although this analog will exert steric restrictions similar to the benzoyl analog, the lone electron pair associated with the central amine may assist formation of a cyclic conformer by forming an intramolecular hydrogen bond. Also, because of increased freedom of rotation which exists about the bonds unlike that for the corresponding amide, the amine may be able to more easily adopt the required comformation.

The Evidence: Hexahydropyrimidines

^{1}H-NMR were considered in order to show that the polyamines do indeed form cyclic conformers; however, it was clear this would not definitively indicate whether or not the L1210 cell polyamine transport apparatus transports six- and/or seven-membered hydrogen-bonded cyclic structures; thus, the importance of cyclic conformers in uptake had to be evaluated by

TABLE 17
Infrared Data for Terminal Diols of the Structure $HO-(CH_2)_n-OH$

n	ν_{free}	ν_{bound}	$\Delta\nu$
2	3644	3612	32
3	3636	3558	78
4	3634	3478	156
5	3638	3485	153
6	3638	3490	148

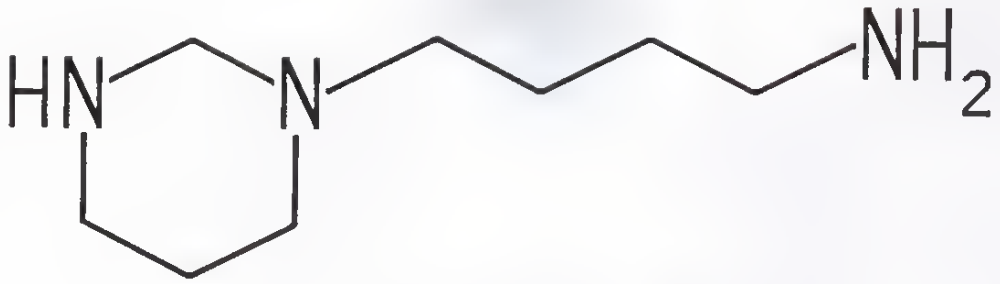
Reference 84.

another method. It was decided to prepare cyclic methylene-bridged analogs of intramolecularly hydrogen bonded norspermidine and spermidine--hexahydropyrimidine analogs, figure 42--and determine if these analogs would be transported by L1210 cells. A hexahydropyrimidine analog is easily synthesized from the appropriate amine and formaldehyde (13, 14). The product still possesses the polyamine backbone intact, but this backbone is "locked in" to a cyclic conformation by the methylene bridge. Since all three nitrogens remain amines, they can still bear positive charge. If cyclic conformers are involved in the uptake mechanism, these analogs should be readily recognized and transported by the polyamine transport apparatus.

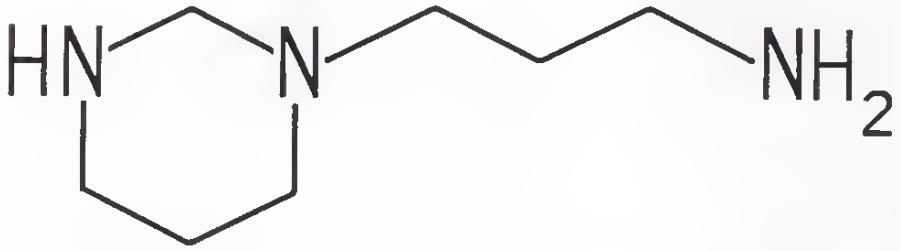
Two questions were adressed relative to these compounds, (1) Are they stable in solution, and (2) Do they compete for the polyamine transport apparatus? The stability was questioned simply because of the potentially reversible nature of hexahydropyrimidine formation. The question was answered utilizing ^{1}H-NMR. A deuterated version of the culture medium employed in the polyamine uptake studies (RPMI-1640) was prepared. N-(4-aminobutyl)-hexahydropyrimidine was added to the medium and a 300MHz ^{1}H-NMR spectrun recorded both immediately and four hours later. A singlet characteristic of the hexahydropyrimidine methylene bridge exhibits a chemical shift of 3.4 ppm downfield from TMS. The two spectra indicated that the methylene bridge of ABHHP was stable during the course of the biological studies. Since the mechanism of hydrolysis is the same for ABHHP and N-(3-aminopropyl)hexahydropyrimidine (APHHP), it was deemed unnecessary to repeat the experiment for the latter.

Once having established the stability of hexahydropyrimidines under experimental conditions, spermidine competition studies were initiated. Briefly, L1210 cells in log growth phase were exposed to ^{14}C-labelled spermidine at concentrations varying from 0.2 to 10 μM in the absence or presence of 10 μM hexahydropyrimidine analog. ^{14}C-spermidine cellular uptake was measured using scintillation procedures. The data were plotted according to the Hofstee method, figure 43, and the K_i's calculated from the equation

$$K_{apparent} = K_m(1 + [i]/K_i),$$



ABHHP



APHHP

FIGURE 42
Cyclic Spermidine Analogs.

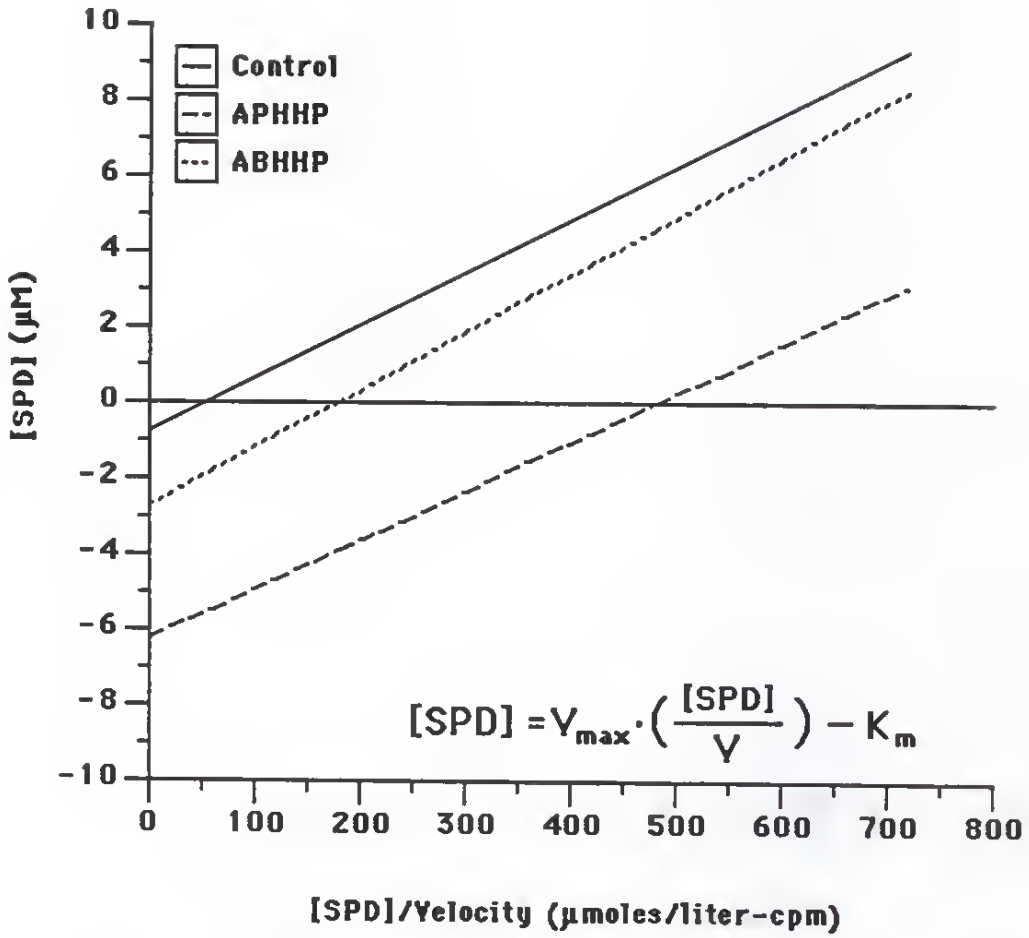


FIGURE 43
Hofstee Plot for Competitive Inhibition of ^{14}C-Spermidine Uptake by Cyclic Polyamine Analogs. Cells Were Incubated for 20 Minutes at 37 Degrees with 0.0, 2.0, 4.0, 6.0, 8.0, or 10.0 µM [^{14}C]-Spermidine and 10.0 µM Analog.

utilizing a K_m of 0.77 μM for spermidine, table 18. The magnitude of K_m for spermidine and K_i for norspermidine are somewhat different than those reported by C. W. Porter et al. (8). This difference can be attributed to the fact that our experiments utilized L1210 cells from tissue culture whereas previous work employed Ascites cells. However, in order to verify that the variance could not be assigned to the fact that we employed different concentrations of polyamines in our studies, a Dixon plot was generated, using norspermidine as a model. The data was plotted using a rearrangement of the Michealis-Menton equation for competitive inhibition

$$\frac{1}{V} = \left[\frac{K_m}{S \cdot V_m \cdot K_i}\right] \cdot [i] + \left[\frac{S + K_m}{S \cdot V_m}\right]$$

The linearity of this plot, figure 44, suggests that the mode of inhibition is the same over the concentration range examined; i.e., K_i is constant.

The ability of the hexahydropyrimidines to prevent the growth of L1210 cells was also measured. These analogs were found to be very effective at preventing cell division, as evidenced by their IC_{50} values, table 18. This may be due to interference with or disruption of polyamine metabolism or function. Whatever the mode of action, it is clear from these data that the possibility of polyamines adopting cyclic conformations during transport is quite reasonable.

TABLE 18
Summary of *In Vitro* Experimental Results for Cyclic Spermidine Analogs.

Compound	Percent of Control[a]	SPD uptake inhibition	
		K_i(μM)[b]	IC_{50}(μM)[c]
none	100	---	---
NSPD	29	4.67 ± 0.33	0.7
APHHP	58.9	3.91 ± 2.57	0.5
SPD	9.5	0.77 ± 0.31(K_m)	1000.0[d]
ABHHP	11.4	1.41 ± 1.15	40.0

[a]Cells were incubated for 20 minutes at 37°C with 10 μM [^{3}H]-spermidine plus100 μM analog.
[b]Prewarmed L1210 cells($5x10^6$) were incubated for 20 min in 1 mL of RPMI-1640 media containing 2 percent Hepes-Mops and 0.2, 0.5, 1.0, 2.0, 5.0, or 10.0 μM [^{3}H]-spermidine and 10 or100 μM analog.
[c]3×10^4 cells/mL were incubated for 48 hours at 37°C with 0 to 100 μM polyamine.
[d]Reference 8.

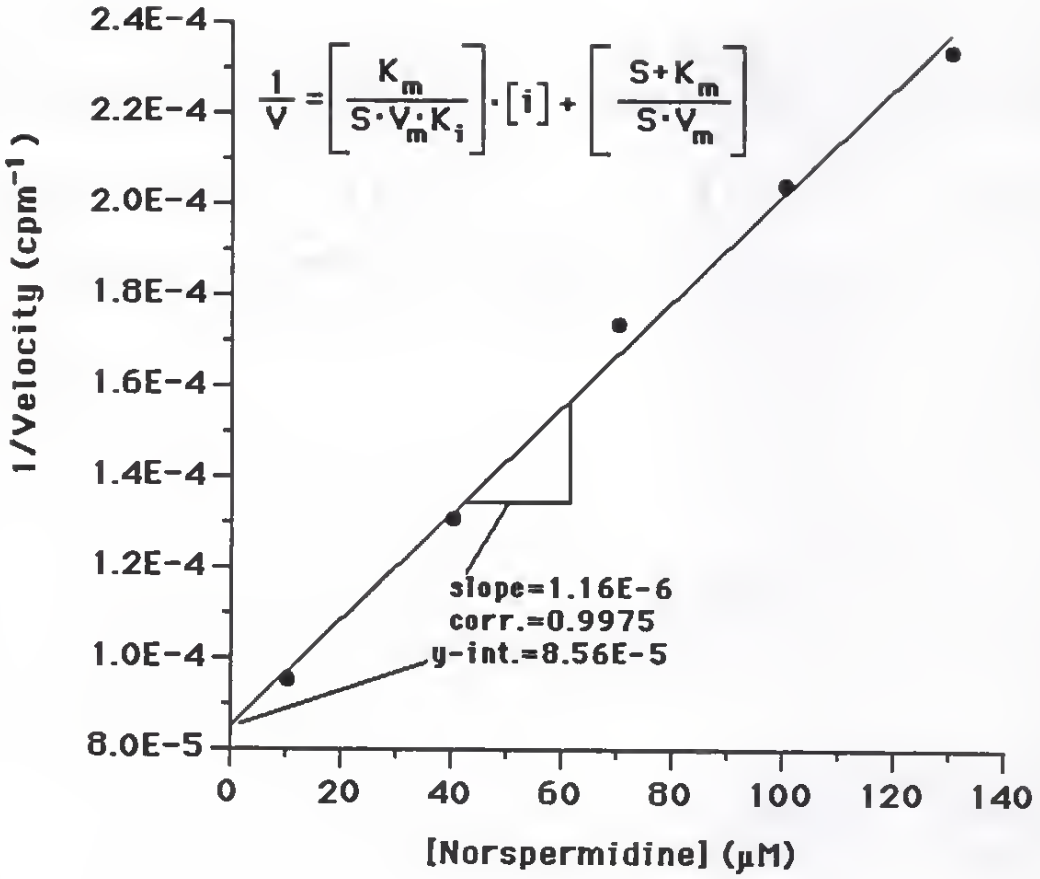


FIGURE 44
Dixon Plot for Norspermidine, a Representative Competitive Inhibitor of ^{14}C-Spermidine Uptake. Cells Were Incubated for 20 Minutes at 37 Degrees with 10 µM [^{14}C]-Spermidine and 10, 40, 70, 100, or 130 µM Norspermidine.

CHAPTER IV

EXPERIMENTAL DETAILS

Synthetic Procedures

N^1,N^7-Bis(2,3-dimethoxybenzoyl)-N^4-succinyl norspermidine (3a)

A solution of N^1,N^7-bis(2,3-dimethoxybenzoyl) norspermidine (4.95 g, 10.8 mmoles), triethylamine (5.4 g, 53 mmoles), and succinic anhydride (1.23 g, 13 mmoles) in 200 mL of CH_2Cl_2 was refluxed for four hours prior to addition of 3-(dimethylamino)propylamine (0.31 g, 3.0 mmoles). The resulting mixture was refluxed overnight. After cooling the reaction mixture was washed with 1M HCl (5 x 20 mL) and the combined aqueous washings were back-extracted with CH_2Cl_2 (25 mL). The combined organic layers were dried with Na_2SO_4, filtered, and evaporated to afford 5.37 g (88 percent) crude product, which separated on silica gel (400 g, 10 percent MeOH:$CHCl_3$) to yield 4.03 g (65 percent) pure product. ^{1}H-NMR ($CDCl_3$) δ 1.6-2.1 (m, 4H), 2.65 (s, 4H), 3.2-3.6 (m, 8H), 3.9 (s,12H),6.9-8.4 (m,8H), 10.6 (br, 1H). An analytical sample was prepared by loading 100mg of product onto silica gel (15g, 12-25 percent MeOH:EtOAc). Analysis calculated for $C_{28}H_{37}N_3O_9 \cdot H_2O$, Calc. 58.22 %C, 6.81 %H, 7.27 %N, found 58.44 %C, 6.73 %H, 7.24 %N.

N^1,N^9-Bis(2,3-dimethoxybenzoyl)-N^5-succinyl homospermidine (3b)

3b was prepared and purified in the same manner as 3a (65 percent). ^{1}H-NMR ($CDCl_3$) δ 1.4-1.9 (m, 8H), 2.55 (s, 4H), 3.2-3.7 (m, 8H), 3.95 (s,12H), 6.9-8.2 (m,8H), 10.6 (br, 1H). Analysis calculated for $C_{30}H_{41}N_3O_9 \cdot H_2O$, Calc. 59.49 %C, 7.16 %H, 6.94 %N, found 59.30 %C, 7.13 %H, 6.92 %N.

N^1,N^7-Bis(2,3-dimethoxybenzoyl)-N^4-glutaryl norspermidine (3c)

3c was prepared and purified in the same manner as 3a (65 percent). ^{1}H-NMR ($CDCl_3$) δ 1.6-2.2 (m, 6H), 2.2-2.8 (m, 4H), 3.2-3.8 (m, 8H), 3.95 (s,12H),7.0-8.3 (m,8H), 10.6 (br, 1H). Analysis calculated for $C_{29}H_{39}N_3O_9 \cdot H_2O$, Calc. 58.87 %C, 6.99 %H, 7.10 %N, found 58.78 %C, 7.05 %H, 7.05 %N.

N^1,N^9-Bis(2,3-dimethoxybenzoyl)-N^5-glutaryl homospermidine (3d)

3d was prepared and purified in the same manner as 3a (65 percent). ^{1}H-NMR ($CDCl_3$) δ 1.4-1.9 (m, 8H), 2.3-2.6 (m, 6H), 3.2-3.7 (m, 8H), 3.95 (s,12H), 6.9-8.2 (m,8H), 10.5 (br, 1H). Analysis calculated for $C_{31}H_{43}N_3O_9 \cdot H_2O$, Calc. 60.08 %C, 7.32 %H, 6.78 %N, found 60.05 %C, 7.31 %H, 6.78 %N.

Bis[[N, N-bis (2,3-dimethoxybenzoyl-3-aminopropyl)] succinamide (2a)

A solution of N^1,N^7-bis(2,3-dimethoxybenzoyl) norspermidine (2.81 g, 5.96 mmoles) and triethylamine (0.9 g, 8.9 mmoles) in 150 mL CH_2Cl_2 was stirred at 0°C under a nitrogen atmosphere. Dropwise addition of succinyl dichloride (0.45 g, 2.90 mmoles) in 50 mL CH_2Cl_2 was completed before allowing the reaction mixture to warm slowly to room temperature and stirring for 24 hours. After cooling to 0°C, 3N HCl (75 mL) was added. Thirty minutes later the organic phase was washed with 3N HCl (3 x 75 mL), dried over Na_2SO_4, filtered, and evaporated to yield 2.88 g (97 percent) crude product, which was chromatographed on silica gel (150 g, 10:45:45 percent MeOH:EtOAc:$CHCl_3$) to yield 1.98 g (85 percent) of pure product. An analytical sample was prepared by HPLC on silica using the same solvent system. ^{1}H-NMR ($CDCl_3$) δ 1.5-2.2 (m, 8H), 2.2-2.8 (m, 4H), 3.0-3.7 (m, 16H), 3.8 (s, 24H), 6.7-8.3 (m,16H). Analysis calculated for $C_{52}H_{68}N_6O_{14}$, Calc. 62.39 %C, 6.85 %H, 8.39 %N, found 62.13 %C, 6.89 %H, 8.34 %N.

Bis[N, N-bis(2,3-dimethoxybenzoyl-4-aminobutyl)] succinamide (2b)

2b was prepared and purified in the same manner as 2a (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.3-1.8 (m, 16H), 2.1-2.8 (m, 4H), 3.0-3.7 (m, 16H), 3.8 (s, 24H), 6.8-8.1 (m, 16H). Analysis

calculated for $C_{56}H_{70}N_6O_{14}$, Calc. 63.62 %C, 7.25 %H, 7.95 %N, found 63.66 %C, 7.29 %H, 7.95 %N.

Bis[N-(2,3-dimethoxybenzoyl-4-aminobutyl)-N- (2,3-dimethoxybenzoyl-3-aminopropyl)] succinamide(2c)

2c was prepared and purified in the same manner as 2a (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.2-2.1 (m, 12H), 2.1-2.9 (m, 4H), 2.9-3.6 (m, 16H), 3.7 (s, 24H), 6.6-8.1 (m, 16H). Analysis calculated for $C_{54}H_{72}N_6O_{14}\cdot H_2O$, Calc. 61.94 %C, 7.12 %H, 8.03 %N, found 61.74 %C, 7.15 %H, 7.92 %N.

Bis[N, N-bis (2,3-dimethoxybenzoyl-3-aminopropyl)] glutaramide (2d)

2d was prepared and purified in the same manner as 2a (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.5-2.1 (m, 8H), 2.1-2.6 (m, 6H), 3.0-3.7 (m, 16H), 3.8 (s, 24H), 6.7-8.3 (m, 16H). Analysis calculated for $C_{53}H_{70}N_6O_{14}$, Calc. 62.71 %C, 6.95 %H, 8.28 %N, found 62.51 %C, 7.03 %H, 8.17 %N.

Bis[[N, N-bis (2,3-dimethoxybenzoyl-4-aminobutyl)] glutaramide (2e)

2e was prepared and purified in the same manner as 2a (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.3-1.8 (m, 16H), 2.1-2.6 (m, 6H), 3.8 (s, 24H), 6.8-8.1 (m, 16H). Analysis calculated for $C_{57}H_{78}N_6O_{14}\cdot 2H_2O$, Calc. 61.83 %C, 7.46 %H, 7.59 %N, found 61.92 %C, 7.21 %H, 7.59 %N.

Bis[N-(2,3-dimethoxybenzoyl-4-aminobutyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)] glutaramide (2f)

2f was prepared and purified in the same manner as 2a (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.2-2.1 (m, 12H), 2.1-2.6 (m, 6H), 2.9-3.6 (m, 16H), 3.7 (s, 24H), 6.6-8.1 (m, 16H). Analysis calculated for $C_{55}H_{74}N_6O_{14}\cdot 2H_2O$, Calc. 61.21 %C, 7.28 %H, 7.79 %N, found 61.11 %C, 6.90 %H, 7.66 %N.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dimethoxybenzoyl- 4-aminobutyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)]succinamide(2g)

A soluton of 3c (1.0 g, 1.74 mmoles), dicyclohexylcarbodiimide (0.38 g, 1.85 mmoles), 4-(dimethylamino)pyridine (20 mg), and N^1,N^8-bis(2,3- dimethoxybenzoyl)spermidine (1.02 g,

2.09 mmoles) in CH_2Cl_2 (150 mL) was stirred for 24 hours. The reaction mixture was filtered and evaporated to afford 1.97g crude oil which was purified b silica gel chromatography (20 g, eluted with 0:50:50 to 10:45:45 EtOH: EtOAc:$CHCl_3$) to yield 1.68 g of pure product (85 percent). An analytical sample was prepared by HPLC on silica using the same solvent system. ^{1}H-NMR ($CDCl_3$) δ 1.5-2.2 (m, 10H),2.65 (s, 4H), 3.1-3.7 (m, 16H), 3.8 (s, 24H),6.9-7.7 (m,12H). Analysis calculated for $C_{53}H_{70}N_6O_{14}\cdot H_2O$, Calc. 61.61 %C, 7.02 %H, 8.13 %N, found 61.54 %C, 7.19 %H, 7.84 %N.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dimethoxybenzoyl-4-aminobutyl)] succinamide(2h)

2h was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.5-2.2 (m, 12H), 2.65 (s, 4H), 3.1-3.7 (m, 16H), 3.85 (s, 24H), 6.9-7.7 (m, 12H). Analysis calculated for $C_{54}H_{72}N_6O_{14}\cdot H_2O$, Calc. 61.94 %C, 7.12 %H, 8.03 %N, found 61.83 %C, 7.14 %H, 8.03 %N.

N-[Bis(2,3-dimethoxybenzoyl-4-aminobutyl)]-N'-[N-(2,3-dimethoxybenzoyl-4-aminobutyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)]succinamide(2i)

2i was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.5-2.2 (m, 14H), 2.65 (s, 4H), 3.1-3.7 (m, 16H), 3.85 (s, 24H), 6.9-7.7 (m, 12H). Analysis calculated for $C_{55}H_{74}N_6O_{14}$, Calc. 63.32 %C, 7.15 %H, 8.06 %N, found 63.38 %C, 7.32 %H, 7.89 %N.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dimethoxybenzoyl-4-aminobutyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)]glutaramide(2j)

2j was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.2-2.1 (m, 10H), 2.1-2.7 (m, 6H), 3.1-3.8 (m, 16H), 3.85 (s, 24H), 6.8-7.8 (m, 12H). Analysis calculated for $C_{54}H_{72}N_6O_{14}$, Calc. 63.02 %C, 7.05 %H, 8.17 %N, found 62.94 %C, 6.93 %H, 8.25 %N.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dimethoxybenzoyl-4-aminobutyl)] glutaramide(2k)

2k was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.4-2.1 (m, 12H), 2.2-2.5 (m, 6H), 3.1-3.7 (m, 16H), 3.9 (s, 24H), 6.9-7.7 (m, 12H). Analysis calculated for $C_{55}H_{74}N_6O_{14}\cdot H_2O$, Calc. 62.25 %C, 7.22 %H, 7.92 %N, found 62.36 %C, 7.21 %H, 7.88 %N.

N-[Bis(2,3-dimethoxybenzoyl-4-aminobutyl)]-N'-[N-(2,3-dimethoxybenzoyl-4-aminobutyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)]glutaramide(2l)

2l was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.4-2.1 (m, 14H), 2.2-2.5 (m, 6H), 3.1-3.7 (m, 16H), 3.9 (s, 24H), 6.9-7.7 (m, 12H). Analysis calculated for $C_{56}H_{76}N_6O_{14}$, Calc. 63.62 %C, 7.25 %H, 7.95 %N, found 63.71 %C, 7.12 %H, 7.81 %N.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dimethoxybenzoyl-4-aminobutyl)] adipamide(2m)

2m was prepared and purified in the same manner as 2g (85 percent). ^{1}H-NMR ($CDCl_3$) δ 1.7 (br, 14H), 2.45 (br, 4H), 3.5 (t, 16H), 3.9 (d, 24H), 7.1 (q, 8H), 7.75 (m, 4H), 8.1 (br, 3H), 8.4 (t, 1H). Analysis calculated for $C_{56}H_{76}N_6O_{14}\cdot H_2O$, Calc. 62.55 %C, 7.31 %H, 7.82 %N, found 62.84 %C, 7.31 %H, 7.81 %N.

N,N'-Bis[bis(2,3-dihydroxybenzoyl-3-aminopropyl)] succinamide(1a)

A solution of 2a (1.2 g, 1.16 mmoles) in CH_2Cl_2 (100 mL) was stirred under nitrogen at 0°C. A soluton of BBr_3 (25 mmoles) in CH_2Cl_2 (50 mL) was addded dropwise. After stirring at room temperature for 16 hours, the reacton vessel was cooled to 0°C and ice cold H_2O (20 mL) was added slowly with vigorous stirring. After stirring for 2 hours, the crude material was filtered and washed altematingly with H_2O and CH_2Cl_2. The resultant solid was dissolved in MeOH and evaporated several times to afford 1.3 g of crude product. This material was chromatographed on Sephadex LH-20 (120 g, eluted with 32 percent EtOH:benzene) to yield 0.98 g product (90 percent). An analytical sample was prepared by HPLC, eluting with 25 percent 0.4F phosphate buffer, pH 3.0 : 75 percent acetonitrile. The desired fractions were placed under a stream of

nitrogen gas to remove the acetonitrile, extracted with EtOAc (3 x 3 mL per fraction), dried over Na_2SO_4, and evaporated. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 8H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{44}H_{52}N_6O_{14}$, Calc. 59.45 %C, 5.90 %H, 9.45 %N, found 59.42 %C, 5.93 %H, 9.41 %N.

N,N'-Bis[bis(2,3-dihydroxybenzoyl-4-aminobutyl)] succinamide (1b)

1b was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 16H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{48}H_{60}N_6O_{14}\cdot H_2O$, Calc. 59.86 %C, 6.49 %H, 8.73 %N, found 59.90 %C, 6.31 %H, 8.60 %N.

N,N'-Bis[bis(2,3-dihydroxybenzoyl-4-aminobutyl)] succinamide (1c)

1c was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 12H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{46}H_{56}N_6O_{14}$, Calc. 60.25 %C, 6.16 %H, 9.16 %N, found 60.08 %C, 6.24 %H, 9.08 %N.

N,N'-Bis[bis (2,3-dihydroxybenzoyl-3-aminopropyl)] glutaramide (1d)

1d was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 8H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{45}H_{56}N_6O_{14}\cdot H_2O$, Calc. 58.69 %C, 6.13 %H, 9.13 %N, found 58.50 %C, 6.24 %H, 8.89 %N.

N,N'-Bis[bis (2,3-dihydroxybenzoyl-4-aminobutyl)] glutaramide (1e)

1e was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 16H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{49}H_{62}N_6O_{14}$, Calc. 61.37 %C, 6.52 %H, 8.76 %N, found 61.33 %C, 6.53 %H, 8.74 %N.

N,N'-Bis[N-(2,3-dihydroxybenzoyl-4-aminobutyl)-N'-(2,3-dihydroxybenzoyl-3-aminopropyl)] glutaramide (1f)

1f was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 12H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{47}H_{58}N_6O_{14}\cdot H_2O$, Calc. 59.48 %C, 6.37 %H, 8.86 %N, found 59.39 %C, 6.39 %H, 8.80 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dihydroxybenzoyl-4-aminobutyl)-N-(2,3-dihydroxybenzoyl-3-aminopropyl)] succinamide(1g)

1g was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 10H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{45}H_{54}N_6O_{14}$, Calc. 59.86 %C, 6.03 %H, 9.31 %N, found 59.62 %C, 6.24 %H, 9.38 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dihydroxybenzoyl-4-aminobutyl)] succinamide(1h)

1h was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 12H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{46}H_{56}N_6O_{14}\cdot H_2O$, Calc. 59.09 %C, 6.25 %H, 8.99 %N, found 59.21 %C, 6.48 %H, 9.21 %N.

N-[Bis(2,3-dihydroxybenzoyl-4-aminobutyl)]-N'-[N-(2,3-dihydroxybenzoyl-4-aminobutyl)-N-(2,3-dihydroxybenzoyl-3-aminopropyl)] succinamide(1i)

1i was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 14H), 2.7 (s, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{47}H_{58}N_6O_{14}\cdot H_2O$, Calc. 59.48 %C, 6.37 %H, 8.86 %N, found 59.62 %C, 6.48 %H, 8.69 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dihydroxybenzoyl-4-aminobutyl-N-(2,3-dihydroxybenzoyl-3-aminopropyl)] glutaramide(1j)

1j was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 10H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{46}H_{56}N_6O_{14}\cdot H_2O$, Calc. 59.09 %C, 6.25 %H, 8.99 %N, found 58.97 %C, 6.09 %H, 9.08 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dihydroxybenzoyl-4-aminobutyl)] glutaramide(1k)

1k was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 12H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{47}H_{58}N_6O_{14}\cdot 2H_2O$, Calc. 58.38 %C, 6.46 %H, 8.69 %N, found 58.29 %C, 6.41 %H, 8.46 %N.

N-[Bis(2,3-dihydroxybenzoyl-4-aminobutyl)]-N'-[N-(2,3-dihydroxybenzoyl-4-aminobutyl)-N-(2,3-dihydroxybenzoyl-3-aminopropyl)]glutaramide(1l)

1l was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 14H), 2.0-2.6 (m, 6H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{48}H_{60}N_6O_{14}\cdot H_2O$, Calc. 59.86 %C, 6.49 %H, 8.73 %N, found 59.93 %C, 6.50 %H, 8.73 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[bis(2,3-dihydroxybenzoyl-4-aminobutyl)] adipamide (1m)

1m was prepared and purified in the same manner as 1a. ^{1}H-NMR (CD_3OD) δ 1.60(br, 12H), 1.8 (br, 4H), 2.35 (br, 4H), 3.4 (m, 16H), 6.6-7.4 (m, 12H). Analysis calculated for $C_{48}H_{60}N_6O_{14}\cdot H_2O$, Calc. 59.86 %C, 6.49 %H, 8.73 %N, found 59.87 %C, 6.52 %H, 8.48 %N.

N,N'-Bis[bis(N-BOC-3-aminopropyl)] glutaramide (4d)

To a solution of N^1,N^7-bis(butyloxycarbonyl)norspermidine hydrochloride (1.95 g, 4.94 mmoles), triethylamine (1.46 g, 14.4 mmoles) and N,N-(dimethyl) aminopyridine (60 mg) in CH_2Cl_2 (150 mL) was added glutaryl dichloride (.38 g, 2.25 mmoles). This solution was stirred at room temperature for 12 hours before transferring to a separatory funnel with CH_2Cl_2 (300 mL) and washing with 1M HCl (200 mL) and 10 percent $NaHCO_3$ (200 mL) to yield 1.97 g crude product (87 percent) ^{1}H-NMR ($CDCl_3$) δ 1.45 (s, 36H), 2.0 (t, 4H), 2.4 (m, 8H), 2.75 (m, 2H), 3.2 (m, 16H). Analysis calculated for $C_{37}H_{70}N_6O_{10}$, Calc. 58.55 %C, 9.30 %H, 11.07 %N, found 58.61 %C, 9.28 %H, 11.22 %N. This compound was exposed to TFA at 0°C for 20 min. to yield the corresponding tetraamine which was used immediately.

2,3-Dimethoxy-5-nitrobenzoyl chloride

To 2,3-dimethoxy-5-nitro benzoic acid (1.92 g, 8.45 mmoles)--synthesized by the method of Cain and Simonsen (84)--was added thionyl chloride (50 mL). The solution was refluxed for 5 hours before cooling to room temperature. Excess thionyl chloride was evaporated and benzene (200 mL) was added. This solution was washed with 13 percent K_2CO_3 (2 x 50 mL), dried over Na_2SO_4, and evaporated to yield 1.98 g product (92 percent). ^{1}H-NMR ($CDCl_3$) δ 3.8 (s, 6H), 7.0 (m, 2H).

N-hydroxysuccinimyl(2,3-dimethoxy-5-nitro)benzoate

To a solution of 2,3-dimethoxy-5-nitro benzoic acid (.68 g, 3.0 mmoles) and N-hydroxysuccinimide (.40 g, 3.5 mmoles) in THF (25 mL) was added DCC (.74 g, 3.6 mmoles). The reaction was stirred 20 hours before filtration and evaporation to yield 1.05 g product (100 percent), which was used without further purification.

N,N-diethyl (2,3-dimethoxy-5-nitro)benzamide

To a solution of diethylamine (.37 g, 5.05 mmoles) in CH_2Cl_2 (25 mL) at 0°C was added dropwise (2,3-dimethoxy-5-nitro)benzoyl chloride (.50 g, 2.03 mmoles) in CH_2Cl_2 (25 mL). This solution was stirred at room temperature for 24 hours before washing with 3 M HCl (2 x 50 mL) to yield 0.58 g crude material (97 percent), which was chromatographed (15 g silica eluted with $CHCl_3$) to yield the pure material (80 percent). ^{1}H-NMR (acetone-d_6) δ 1.1 (t, 3H), 1.2 (t, 3H), 3.1 (q, 2H), 3.5 (q, 2H), 4.0 (d, 6H), 7.6 (d, 1H), 7.8 (d, 1H). Analysis calculated for $C_{13}H_{18}N_2O_5$, Calc. 55.31 %C, 8.43 %H, 9.92 %N, found 55.48 %C, 6.39 %H, 9.90 %N.

N,N-diethyl (2,3-dihydroxy-5-nitro)benzamide

To a solution of BBr_3 (12 mL, 12 mmoles) in CH_2Cl_2 (20 mL) at 0°C was added dropwise N,N-diethyl (2,3-dimethoxy-5-nitro)benzamide (.18 g, .67 mmoles) in CH_2Cl_2 (40 mL). The suspension was stirred at room temperature before adding ice/water (20 mL) and stirring an additional 3 hours. The mixture was then evaporated to an aqueous solution which was extracted with ethyl acetate (8 x 25 mL). This extract was extracted with 10 percent NaOH (3 x 50 mL), acidified with concentrated HCl, and extracted again with ethyl acetate (3 x 75 mL) to yield the crude material, which was chromatographed (2 g silica eluted with 10 percent MeOH:$CHCl_3$) to yield .12 g product (76 percent). ^{1}H-NMR (acetone-d_6) δ 1.1 (t, 6H), 3.5 (q, 2H), 4.1 (q, 2H), 7.79 (s, 2H). Analysis calculated for $C_{11}H_{14}N_2O_5$, calc. 51.97 %C, 5.55 %H, 11.02 %N, found 52.14 %C, 5.69 %H, 11.22 %N.

N^1-(2,3-dimethoxy-5-nitrobenzoyl)-N^3-[3-(2,3-dimethoxy-5-nitro-N-benzoyl) amino-1-propyl]hexahydropyrimidine(8)

To a solution of APHHP (.39 g, 2.7 mmoles) and triethylamine (0.6 g, 5.93 mmoles) in CH_2Cl_2 (40 mL) at 0°C was added 2,3-dimethoxy-5-nitrobenzoyl chloride (1.33 g, 5.41 mmoles). The solution was stirred 1 hour and evaporated. Ethyl acetate (25 mL) was added, causing an impurity to precipitate. After filtration, the mother liquor was chromatographed (85 g silica eluted with EtOAc) to yield 1.24 g product (82 percent). ^{1}H-NMR ($CDCL_3$) δ 1.5-2.1 (m, 4H), 2.8 (m, 4H), 3.5 (m, 4H), 4.1 (s,12H), 4.5 (s, 2H), 7.8 (m, 2H), 8.5 (dd, 2H). Analysis calculated for $C_{25}H_{30}N_5O_{10}$, Calc. 53.57 %C, 5.39 %H, 12.49 %N, found 53.55 %C, 5.41 %H, 12.44 %N.

N-[3-(2,3-dimethoxy-N-benzoyl)amino-1-propyl]hexahydropyrimidine(9)

To a solution of APHHP (132 mg, .92 mmoles) in CH_2Cl_2 (25 mL) at 0°C was added N-hydroxysuccinimyl(2,3-dimethoxy)benzoate (250 mg, .89 mmoles) in CH_2Cl_2 (50 mL) over a 2-hour period before stirring at room temperature for 24 hours. The solution was extracted with 1 M HCl (3 x 25 mL), made basic with NaOH (s), and extracted with CH_2Cl_2 (4 x 25 mL). This crude material was chromatographed (20 g silica eluted with 1 percent triethylamine:MeOH) to yield 100 mg product (45 percent). ^{1}H-NMR ($CDCl_3$) δ 1.55 (quint, 2H), 1.75 (quint, 2H), 2.3 (d, 2H), 2.7 (m, 4H), 3.4 (s, 2H), 3.5 (t, 2H), 3.9 (s, 6H), 7.1 (m, 1H), 7.65 (dd, 1H), 8.3 (t,1H). Analysis calculated for $C_{16}H_{25}N_3O_3 \cdot 2H_2O$, calc. 55.96 %C, 8.51 %H, 12.24 %N, found 55.88 %C, 8.65 %H, 12.44 %N.

N^1-(2,3-dimethoxybenzoyl)norspermidine

A solution of 9 (100 mg, .29 mmoles), piperidine (2 drops), and dimedone (210 mg, 1.5 mmoles) in ethanol (20 mL) was refluxed for 18 hours. After evaporation, ethyl ether (50 mL) and 1 M HCl (20 mL) were added, and the organic layer was extracted with 1 M HCl (2 x 20 mL). The combined aqueous layers were saturated with NaCl(s) and made basic with NaOH(s) before extracting with CH_2Cl_2 (6 x 20 mL) to yield the crude product, which was chromatographed

(5.5 g silica eluted with 2 percent to 10 percent NH_4OH:MeOH) to yield 100 mg product (100 percent). Spectral data were identical to those already reported (84).

N^1-(2,3-dimethoxybenzoyl)-N^7-(2,3-dimethoxy-5-nitrobenzoyl)norspermidine(10)

10 was prepared and purified in the same manner as 9. ^{1}H-NMR ($CDCl_3$) δ 1.3 (t, 4H), 2.2 (m,2H), 2.65 (m, 2H), 3.1 (m, 2H), 3.65 (m, 4H), 3.9-4.1 (m, 12H), 7-8.5 (m, 7H). Analysis calculated for $C_{24}H_{32}N_4O_8 \cdot H_2O$, calc. 55.16 %C, 6.56 %H, 10.72 %N, found 55.15 %C, 6.56 %H, 10.73 %N.

N^1,N^7-(2,3-dimethoxy-5-nitrobenzoyl)norspermidine(6)

A solution of 8 (51 mg, .091 mmoles), piperidine (1 drop), and dimedone (140 mg, 1 mmole) in 50 percent aqueous ethanol (60 mL) was refluxed for 24 hours, evaporated to an aqueous solution, and extracted with CH_2Cl_2 (3 x 30 mL). This crude material was chromatographed (10 g silica eluted with 0 percent to 5 percent NH_4OH:MeOH) to yield 40 mg product (80 percent). This compound was found to be very unstable and was used as soon as isolated. ^{1}H-NMR ($CDCl_3$) δ 1.9 (quint, 4H), 2.6 (s, 1H), 2.8 (t, 4H), 4.0 (d, 12H), 7.8 (d, 2H), 8.2 (t, 2H), 8.45 (d, 2H).

Bis{N, N-bis[(2,3-dimethoxybenzoyl-5-nitro)-3-aminopropyl]} glutaramide(5d)

Method 1. To a solution of 6 (220 mg, .4 mmoles) and triethylamine (90 mg, .89 mmoles) in CH_2Cl_2 (10 mL) at 0°C was added dropwise glutaryl dichloride (32 mg, .19 mmoles) in CH_2Cl_2 (15mL). The reaction was stirred 30 mins. before washing with 1 M HCl (3 x 10 mL) and 1 M NaOH (3 x 15 mL). Preparative chromatography (20 cm x 20 cm silica plate developed with 1:2:7 methanol:chloroform:ethyl acetate and extracted with 50 percent methanol:ethyl acetate) yielded 150 mg product (66 percent). ^{1}H-NMR ($CDCl_3$) δ 1.9 (m, 8H), 2.4 (t, 8H), 3.5 (t, 16H), 4.1 (t, 24H), 7.9 (m, 4H), 8.1 (t, 2H), 8.45 (t, 2H), 8.5 (m, 4H). Analysis calculated for $C_{53}H_6N_{10}O_{22}$, Calc. 53.35 %C, 5.41 %H, 11.74 %N, found 53.21 %C, 5.62 %H, 11.62 %N.

Method 2. To a solution of 4d (210 mg, .26 mmoles) and K_2CO_3 (.44 g, 3.18 mmoles) in dH_2O (15 mL) at 4°C was added dropwise 2,3-dimethoxy-5-nitro-benzoyl chloride (.34 g, 1.36

mmoles) in benzene (25 mL) with vigorous stirring. The reaction was stirred 4 hours before adding CH_2Cl_2 (12 mL) and dH_2O (5 mL). After 30 mins. the mixture was transferred to a separatory funnel with 5 percent cold NaOH (50 mL) and CH_2Cl_2 (50 mL). The aqueous layer was extracted with $MeCl_2$ (2 x 50 mL) to yield 300 mg product (98 percent). Spectral data were the same as those obtained by method 1.

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dimethoxy-5-nitrobenzoyl-3-aminopropyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)succinamide

N-[Bis(2,3-dimethoxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dimethoxy-5-nitro-benzoyl-3-aminopropyl)-N-(2,3-dimethoxybenzoyl-3-aminopropyl)]succinamide was prepared and purified in the same manner as 2g. ^{1}H-NMR ($CDCl_3$) δ 1.9 (br, 6H), 2.3 (br, 2H), 2.7 (s, 4H), 3.5 (t, 16H), 7.0-8.6 (m, 15H). Analysis calculated for $C_{52}H_{67}N_7O_{16}$ calc. 59.70 %C, 6.46 %H, 9.37 %N, found 59.90 %C, 6.49 %H, 9.40 %N.

N-[Bis(2,3-dihydroxybenzoyl-3-aminopropyl)]-N'-[N-(2,3-dihydroxy-5-nitrobenzoyl-3-aminopropyl)-N-(2,3-dihydroxybenzoyl-3-aminopropyl)]succinamide(11a)

11a was prepared and purified in the same manner as 2g. ^{1}H-NMR ($CDCl_3$) δ 1.5 (m, 2H), 2.1 (m, 6H), 2.9 (s, 4H), 3.5 (m, 16H), 6.7-8.4 (m, 11H). Analysis calculated for $C_{44}H_{51}N_7O_{16}$, Calc. 56.69 %C, 5.50 %H, 10.50 %N, found 56.41 %C, 5.63 %H, 10.47 %N.

N,N'-Bis[N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine]-N-BOC-glutamamide (12f)

To a solution of N^1,N^9-bis(2,3-dimethoxybenzoyl) homospermidine (1.16 g, 2.39 mmoles), dicylohexylcarbodiimide (0.50 g, 2.41 mmoles), and N-BOC- glutamic acid (0.28 g, 1.15 mmoles) in CH_2Cl_2 (30 mL) was added 4-(dimethylamino)pyridine (20 mg). The solution was stirred for 12 hours prior to filtraton and evaporation of the filtrate to yield 1.45 g crude oil. This oil was purified by silica gel chromatography (75 g, eluted with 1:8:8 EtOAc:acetone: benzene) to yield 1.05 g product (60 percent). ^{1}H-NMR ($CDCl_3$) δ 1.3 (s, 9H), 1.5-1.8 (m, 16H), 2.3-2.5 (m, 5H), 3.1-3.6 (m, 16H), 3.9 (s, 24H), 4.6 (br, 1H), 5.4 (br, 1H), 6.9-8.2 (m,16H). Analysis calculated for $C_{60}H_{83}N_7O_{16}$, Calc. 62.21 %C, 7.22 %H, 8.46 %N, found 62.00 %C, 7.58 %H, 8.32 %N.

N,N'-Bis[N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine]glutamamide·TFA(13f)

Triflouroacetic acid (10 mL) was cooled to 0°C, added to 12f (0.6 g, 0.51 mmoles) and stirred at 0°C for 60 minutes before evaporating. Benzene (2 x 20 mL) was added and evaporated to yield 0.75 g crude product, which was purified by silica gel chromatography (15 g, packed and loaded with 1:1 EtOAc:$CHCl_3$ and eluted with 15:42.5:42.5 to 20:40:40 EtOH:EtOAc:$CHCl_3$) to yield 0.7 g product (100 percent). ^{1}H-NMR ($CDCl_3$) δ 1.5 (br, 18H), 2.4-2.7 (m, 4H), 3.1-3.5 (m, 16H), 3.9 (s, 24H), 6.9-8.2 (m,16H). Analysis calculated for $C_{57}H_{76}N_7O_{16}F_3$, Calc. 58.40 %C, 6.53 %H, 8.36 %N, found 58.56 %C, 6.79 %H, 8.19 %N.

Bis[N^1,N^8-bis(2,3-dihydroxybenzoyl)spermidine]glutamamide·HBr(25)

25 was prepared and purified in the same manner as compound 1a. ^{1}H-NMR (CD_3OD) δ 1.7 (m, 12H), 2.0-2.65 (m, 4H), 3.45 (m, 16H), 6.6-7.3 (m, 12H). Analysis calculated for $C_{47}H_{60}N_7O_{14}Br$, calc. 54.97 %C, 5.89 %H, 9.55 %N, found 55.09 %C, 5.93 %H, 9.56 %N.

N-BOC-8-aminocaprylic acid

BOC-ON (1.59 g, 6.46 mmoles) was added to a solution of 8-aminocaprylic acid (1.00 g, 6.28 mmoles) and triethylamine (1.81 g, 17.9 mmoles) in tetrahydrofuran (15 mL) and dH_2O (15 mL). This solution was stirred 6 hours before evaporating the acetone. The aqueous layer plus 30 mL H_2O was washed with ethyl ether (3 x 25 mL), acidified with concentrated HCl, and extracted with EtOAc (3 x 25 mL). The combined organic layers were dried with Na_2SO_4 and evaporated to afford 1.65 g of oil (100 percent). ^{1}H-NMR ($CDCl_3$) δ 1.1-1.8 (m, 10H), 1.45 (s, 9H), 2.3 (t, 2H), 3.1 (m, 2H), 4.85 (br,1H), 10.6 (s, 1H). Analysis calculated for $C_{13}H_{26}NO_4$, Calc. 59.97 %C, 10.07 %H, 5.38 %N, found 59.93 %C, 10.10 %H, 5.33 %N.

N-[(8-N-BOC-aminocaprylyl)bis[N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine]]glutamamide(14f)

A solution of 13f (0.49 g, 0.41 mmoles), N-BOC-8-aminocaprylic acid (0.13 g, 0.50 mmoles), dicyclohexylcarbodiimide (0.11 g, 0.53 mmoles) and 4-(dimethylamino)pyridine (10 mg) in ethyl ether (20 mL) and CH_2Cl_2 (2 mL) was stirred 24 hours, filtered, and evaporated to yield 0.65 g of crude product, which was purified on silica gel (60 g, eluted with 10:45:45

EtOH:EtOAc:$CHCl_3$) to yield 0.37 g of oil (69 percent). This oil was dissolved in CH_2Cl_2 (50 mL), washed with saturated $NaHCO_3$ (3 x 25 mL), dried over Na_2SO_4, and evaporated to yield 0.29 g product (55 percent). ^{1}H-NMR ($CDCl_3$) δ 0.8-2.6 (m, 26H), 1.45 (s, 9H), 2.6-3.8 (m, 18H), 3.9 (s, 24H), 4.8 (br, 2H), 7.0-8.2 (m, 12H). Analysis calculated for $C_{68}H_{98}N_8O_{17}$, Calc. 62.85 %C, 7.60 %H, 8.62 %N, found 62.80 %C, 7.67 %H, 8.78 %N.

N-(8-aminocaprylyl)bis[N^1,N^8-bis(2,3-dimethoxybenzoyl)spermidine]glutamamide·TFA (15f)

14f (0.29 g ,0.22 mmoles) was stirred for 20 minutes at 0°C in TFA (10 mL) before evaporating. the remaining oil was dissolved in saturated aqueous NaCl (20 mL), made basic with $NaHCO_3$(s) and extracted with chloroform (5 x 25 mL) to yield 0.22 g product (82 percent). ^{1}H-NMR ($CDCl_3$) δ 1.3 (s, 9H), 1.5 (m, 22H), 2.3 (m, 3H), 3.5 (m, 18H), 3.9 (s, 24H), 6.5-8.3 (m, 17H). Analysis calculated for $C_{63}H_{90}N_8O_{15}$, Calc. 63.09 %C, 7.56 %H, 9.34 %N, found 63.22 %C, 7.60 %H, 9.30 %N.

IRP-64 acid chloride resin(18)

Resin preparation. To IRP-64 (5.8 g) was added thionyl chloride (25 mL) and DMF (1.0 mL) and the mixture was refluxed for 3 hours. After cooling, the resin was washed with dry benzene (100 mL) and dry THF (100 mL) and placed under high vacuum.

Determination of equivalency. To ^{14}C-glycine ethyl ester (33.83 mg, 250 mmoles) was added CH_2Cl_2 (15 mL) and triethylamine (2.5 mL). To each of two flasks was added 18 (1.65 mg and 7.72 mg, respectively) and the prepared solution (2.0 mL). To determine the degree of non-specific binding, to two additional flasks was added IRP-64 (15.04 mg and 12.12 mg, respectively) and the prepared solution (5.0 mL). The samples were mixed overnight and the radioactivity remaining in solution determined by scintillation counting. It was calculated that the equivalency of acid chloride groups was approximately 1.2 meq/gm of resin, about 11 percent conversion from the acid.

IRP-64 methylated resin derivative(21f)

To CH_2Cl_2 (10 mL) and triethylamine (1 mL) was added 13f (50 mg) and 18 (185 mg). The suspension was stirred 6 hours and refluxed an additional 12 hours before adding methanol (1 mL) and refluxing 3 hours. When cool, the suspension was filtered and washed well with CH_2Cl_2 to yield 240 mg product. The equivalency was calculated gravimetrically to be 0.24 meq/gm resin.

IRP-64 catechol resin (24f)

To 21f (77 mg) and CH_2Cl_2 (6 mL) was added BBr_3 (2 mL of a 1.0 M solution in CH_2Cl_2). The mixture was stirred 6 hours. To the cooled flask was added dH_2O (2 mL) with vigorous stirring for 2 hours. The resulting mixture was filtered and washed with CH_2Cl_2 (50 mL), dH_2O (50 mL) and methanol (30 mL) and placed under high vacuum to yield 45 mg of iron-positive product.

CH-Sepharose-4B catecolamide derivative (26)

Phosphate buffer. NaCl (2.9 g) and Na_2HPO_4 (1.76 g) were added to dH_2O (200 mL) and the pH adjusted to 7.2 with H_3PO_4 before diluting with dH_2O to 500 mL. This solution was diluted to 1.0 liter with ethanol.

Acetate buffer. NaCl (14.5 g) and sodium acetate (4.1 g) were added to dH_2O (450 mL), the pH adjusted to 4 with AcOH, and diluted with dH_2O to 500 mL. This solution was passed through Chelex resin and an equal voume of ethanol was added.

Resin preparation. CH-Sepharose 4B (2.09 g) was stirred in 1 mM HCl (30 mL) for 5 minutes. After filtering and washing with more HCl (40 mL) the resin remained in HCl for an additional 15 minutes, at which time it was filtered and washed with phosphate buffer (100 mL).

Resin derivatization. The prepared resin was divided between two tubes. To one tube was added phosphate buffer (7 mL) followed by 25 (76.6 mg) in methanol (.5 mL) and phosphate buffer to 10 mL. To the other tube was added phosphate buffer (10 mL). Both tubes were gently mixed for 24 hours before adding ethanolamine (25 mL) to each tube and

mixing an additional 6 hours. The resins were individually washed with phosphate buffer (50 mL), acetate buffer (50 mL), and again with phosphate buffer (50 mL).

Job's Plots

All ligands and metals were prepared as millimolar solutions. The same buffer prepared for competition studies was used (pH 9.2 ammonia). To each of 11 tubes was added buffer (100 mL), dH_2O (2.8 mL), metal (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 µL), and ligand (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, or 0 µL). The UV-visible absorbance spectra were then recorded. The total molarity of these solutions remained constant at 33.3 µmoles/liter.

Precipitation Techniques

1. To 2.50 mL of dH_2O was added 7.2F NH_3/NH_4Cl buffer, pH 9.2 (100 µL), 0.9073 mM $ThCl_4$ (100 µL), and 1.0 equivalent of a catecholamide ligand (100 µL of methanolic solution). After 1 or 30 minutes the pH was adjusted to neutrality with concentrated HCl (30 µL) and the suspension filtered through a 0.45 µm filter. Next, more HCl (150 µL) and ARS (2.0 mL) were added and the absorbance at 666 nm recorded.

2. A 3.362×10^{-4} M solution of ligand buffered at pH 9.0 (2.7 mL) was acidified (pH 6) and stirred before one equivalent of thorium (100 µL) was added. The suspension was stirred for several minutes, filtered, and concentrated HCl (150 µL) and ARS (2.0 mL) were added and the absorbance at 666 nm recorded.

3. To 2.50 mL of dH_2O was added 7.2F NH_3/NH_4Cl buffer, pH 9.2 (100 µL), 0.9073 mM $ThCl_4$ (100 µL), and concentrated HCl (30 µL). One equivalent of a catecholamide ligand (100 µL of methanolic solution) was added and this acidic solution mixed. The suspension was filtered, and more HCl (150 µL) and ARS (2.0 mL) were added and the absorbance at 666 nm recorded.

Eriochrome Black T Competition

EBT Solution

For accuracy and reproducibility of data, the commercially available indicator grade EBT was purified by a modified method of Robinson and Mills (85). A buffer solution was prepared by adding 150 mL of glacial acetic acid and 250 mL dH_2O to a 500 mL volumetric flask. The pH was adjusted to 4.0 with NaOH prior to diluting to mark with dH_2O. The impure EBT (10 g) was stirred with dH_2O (20 mL) and heated to boiling with constant stirring. The above acetate buffer (20 mL) was added and the mixture allowed to cool. The solution was suction filtered and washed with additional buffer. This process was repeated six times. The resultant solid was boiled with absolute ethanol (40 mL), suction filtered, and washed with hot ethanol (60 mL). This procedure was repeated six times. The solid was dried in the presence of P_2O_5 under high vaccuum at 65°C to yield the pure dye. A purity of 98.7 percent was determined by acid-base titration.

An EBT stock solution was prepared by dissolving 56.77 mg EBT in methanol and diluting to a final volume of 250 mL with methanol (final concentration = 4.922×10^{-4} M). A working solution was prepared by diluting 40 mL stock EBT to 100 mL. (1.969×10^{-4} M).

Hydroxylamine Solution

Hydroxylamine hydrochloride (1.75 g) was weighed into a 250 mL volumetric flask, dissolved in and diluted to mark with dH_2O (final concentration = 0.100 F).

Masking Agent

Into 500 mL dH_2O in a 1.0 liter volumetric flask was dissolved triethanolamine (10.05 g) and disodium EDTA (37.24 g). The solution was diluted to mark, achieving final concentrations of 0.1009 M TEA and 0.1000 M EDTA.

Thorium(IV) Solutions

$^{232}ThCl_4$ (62.43 mg) was added to a 10 mL volumetric flask and diluted to mark with dH_2O (concentration = 1.67×10^{-2} M). 3.00 mL of this solution were transfered to a 10 mL volumetric flask and diluted to mark with dH_2O (final concentration = 5.01×10^{-3} M). A working solution was

prepared by diluting 2.00 mL to 100 mL (1.00×10^{-4} M). The concentration of the stock solution was determined by EDTA titration, using Xylenol Orange as the indicator (86).

Buffer

To a 1.0 liter volumetric flask was added NH_4Cl (60 g), dH_2O (200 mL), and NH_4OH (400 mL). This solution was adjusted to pH 9.2 with concentrated HCl solution before diluting to mark with dH_2O.

Standard Curve

To each of nine 100 mL volumetric flasks was added hydroxylamine solution (1.00 mL), $ThCl_4$ solution (0.00, 2.50, 5.00, 7.50, 10.00, 15.00, 20.00, and 25.00 mL, respectively), masking agent (1.00 mL), and dH_2O (25 mL). After adding EBT solution (5.00 mL) and buffer (3.00 mL), the solutions were diluted to mark with dH_2O and allowed to stand for at least 10 minutes before reading their visible spectra.

Experimental Samples

A solution was prepared as described, with 25 mL of thorium. One equivalent of a ligand was added (100 µL of methanolic solution), and the color was observed for 15 seconds. Development of a blue color during this time was recorded as positive (+).

Competition With Nitrocatecholamides

Stock Solutions

Buffer. 3.0 F NH_3, pH 7.2 in dH_2O.

Catecholamide stock solutions. 1.0 mM in methanol, except for 27 which was prepared in dH_2O.

Thorium stock solution. 9.073×10^{-4} M $^{230}ThCl_4$ in dH_20, as determined by EDTA titration, using Xylenol Orange as the indicator (88).

Copper stock solution. 6.19×10^{-3} M $Cu(OAc)_2$ in dH_20. A working solution was prepared at a concentration of 1.00 mM, and used for subsequent experiments.

Cl-PAN stock solution. 3.00 mM in DMF.

One-Metal-Two-Ligand Competition

4.5 mL polypropylene tubes with snap caps were used. To each tube was added buffer (300 µL), 2Z (100 µL), a catecholamide or methanol (100 µL), copper (0, 20, 40, 60, 80, or 100 µL plus 100, 80, 60, 40, 20, or 0 µL dH_2O), and dH_2O (2.6 mL) under a N_2 atmosphere. Tubes were sealed and mixed 6 hours before recording their visible absorption spectra. Each experiment was performed three times.

Two-Metals-One-Ligand Competition

To each tube was added buffer (300 µL), 1a (0, 18, 36, 54, 72, or 90 µL plus 90, 72, 54, 36, 18, or 0 µL methanol), ^{232}thorium (100 µL), copper (90 µL), Cl-PAN (85 µL), dH_2O (2.65 mL), and benzene (1.3 mL). Tubes were sealed and mixed 24 hours. After sitting for 30-60 minutes, an aliquot of each benzene layer (900 µL) was added to DMF (500 µL), shaken, and the visible absorption spectra recorded. Each experiment was performed three times.

Resin Experiments

Plasma Experiments

Human plasma (30 mL) was filtered (0.2 µm) and combined with ^{230}thorium (45,000 cpm, 0.45 µmole) and mixed for 10 minutes. To each of six tubes was transferred 4.5 mL of spiked plasma. Two tubes were empty prior to the addition, two contained control resin (2-6 mg), and two tubes contained catecholamide resin (2-6 mg). These samples were sealed and mixed for 24 hours at 37°C. Each sample was filtered (0.45 µm) and counted by liquid scintillation (500 µL sample plus 10 mL Aquasol 2).

Biological Evaluation

Urinary Clearance

^{230}Thorium (100,000 cpm in 0.09 M citrate) was administered intraperitoneally. One hour later, chelators (100 or 200 µmoles/kg in 40 percent Chremophor/PBS) were injected intraperitoneally. Animals were placed in metabolic cages with food and water *ad libitum*. Urine samples were collected for 6 hour intervals.

Gall Bladder Excision

Thirty-six mice (A/J) weighing 28-32 grams were given ^{230}thorium (50,000 cpm in 0.09 M citrate) by intraperitoneal injection. One hour later, the mice received vehicle (50 percent Chremophor/PBS) or 1f (200 µmoles/kg) intraperitoneally. Three mice from each group were sacrificed after 3, 6, 12, 24, 36, and 48 hours, and their gall bladders were removed and digested in tissue solubilizer (1 mL Soluene 350, Packard). After 24 hours, hydrogen peroxide (0.5 mL, 30 percent) and ethanol (0.5 mL) were added. Scintillation fluid (5 mL Aquasol 2) was added to each sample, and the thorium content measured by liquid scintillation counting.

Bile Duct Cannulation

Chronic cannulae were placed in the bile ducts of adult male Sprague-Dawley rats, allowing for continuous collection of bile. The cannula emerges from the incision and, using a skin-tunneling needle, is directed under the skin to behind the rat's neck. The emerging cannula is directed through a torque-transmitting spring tether to a fluid swivel located above the metabolic cage. The animal is able to move about freely in the cage while urine and bile were collected. After completing the cannulation procedure, rats were given ^{230}thorium (50,000 cpm in 0.5 mL 0.09 M citrate) by intraperitoneal injection. One hour later, chelators (100 or 200 µmoles/kg in 40 percent Chremophor/PBS) were administered intraperitoneally. Bile was collected for 3 hour intervals for 48 hours.

Potentiometric Measurements

A 20.00 mL sample which contained 0.1 M KCl and approximately 0.001 M polyamine was placed in a water-jacketed cell and titrated with 1.06 M HCl at 25°C using a Radiometer-Copenhagen DTS833 Digital Titration System in conjunction with a Lauda K-2/R circulating constant temperature bath. The data were analysed by the computer program PHFIT (76). Each polyamine was titrated at least five times.

Stability Studies

To the culture medium (RPMI-1640 containing 2 percent HEPES-MOPS buffer, 4.0 mL) was added APHHP (24.6 mg). This solution was adjusted to the same pH as the original medium (7.5) and lyophylized and reconstituted with D_2O several times. The resultant solution was 43 mM in ABHHP. 300MHz ^{1}H-NMR spectra were recorded starting at the time of reconstitution and continuing for three hours. A characteristic singlet appears at 3.4 ppm downfield from TMS, and was monitored throughout the experiment. These spectra indicated that the hexahydropyrimidine moiety remained intact during this time period.

Inhibition of Spermidine Uptake

L1210 cells were maintained in logarithmic growth phase to a density of approximately 8 x 10^5cells/mL in RPMI-1640 tissue culture medium containing 2 percent 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-3(N-morpholino)propane sulfonic acid, 1.0 mM aminoguanidine, and 10 percent fetal calf serum before centrifugation at 500 g at 4°C for 7 minutes. The pellet was resuspended with cold culture medium to a density of 10^7 cells/mL. To each of four vessels containing ^{14}C-spermidine and spermidine analog in 500 µL culture medium was added 500 µL of 10^7 cells/mL. Three of the samples were prewarmed to 37°C and shaken at 37°C for 20 minutes, while the other was not prewarmed and was incubated at 0°C to measure non-specific binding. All samples were then centrifuged at 500g for 5 minutes at 0°C and the supernatant discarded. The pellet was washed twice with 5mL of cold culture medium containing 1.0 mM spermidine to displace non-specifically bound ^{14}C-spermidine. The pellet was dissolved in 300

µL of 1.0 N NaOH at 60°C for 60 minutes and acidified with 700 µL 1.0 N HCl. 800 µL was transferred to a scintillation vial for counting.

Percent uptake

L1210 cells were exposed to 10 µM ^{14}C-spermidine and 100 µM spermidine analog, and the inhibited uptake is expressed as the percent of uninhibited uptake. Each experiment was performed at least four times.

Hofstee plots

Cells were exposed to 10 µM spermidine analog and ^{14}C-spermidine at concentrations varying from 0.2 to 10.0 µM. The results are expressed as an inhibition constant (K_i) for the polyamine uptake apparatus. Each experiment was performed at least four times.

Dixon plot

Cells were exposed to 10 µM ^{14}C-spermidine and norspermidine at concentrations varying from10.0 to 130.0 µM. The results are expressed as a correlation coefficient. The experiment was performed three times.

IC_{50} Measurements

L1210 cells were maintained in logarithmic growth in RPMI-1640 tissue culture medium containing 2 percent 4-(2-hydroxyethyl)-1-piperazineethanesulfonicacid-3(N-morpholino)-propanesulfonic acid, 1.0 mM aminoguanidine, and 10 percent fetal calf serum. Cells (50mL) were grown in 75 cm^2 tissue culture flasks at 37°C. Cells were treated while in logarithmic growth (3 x 10^4 cells/mL) with spermidine analogs. After 48 hours, cells were counted and the IC_{50} measured. Each experiment was performed at least four times.

SUMMARY AND CONCLUSIONS

This work has described the diverse applications of hexahydropyrimidines, both as biologically active molecules and as a reagent for the selective acylation of triamines, leading to the preparation of a wide variety of organic molecules. Hexahydropyrimidines have been shown to compete very effectively for uptake by the polyamine transport apparatus, supporting the idea that a cyclic conformation, assisted by intramolecular hydrogen bonding, is an important part of substrate recognition. These hexahydropyrimidines were also shown to be effective inhibitors of L1210 cell proliferation in vitro.

In addition, hexahydropyrimidines served as intermediates in the synthesis of H-shaped octacoordinate catecholamide chelators. These chelators were shown to bind actinides selectively in solution, and to selectively precipitate actinides from solution, effectively decontaminating the solution.

A resin-bound octacoordinate chelator was synthesized and shown to be able to decontaminate aqueous solutions as effectively as the soluble chelator. This resin-bound chelator was able to chelate the protien-bound actinides present in human plasma, as well.

A nitro derivative of a catecholamide chelator was synthesized. This derivative was utilized to measure the conditional formation constant for the complex formed between an H-shaped ligand and thorium(IV), and the stoichiometry of these complexes.

H-shaped ligands were shownto be non-toxic in mice and rats, and were able to stimulate excretion of a large portion of an injected actinide burden, mainly via the feces.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Raymond J. Bergeron, Chairman
Professor, Medicinal Chemistry

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Richard R. Streiff
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Merle A. Battiste
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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This dissertation was submitted to the Graduate Faculty of the College of Pharmacy and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August 1987

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